Remarks

The amendments

New claims 33 and 34 have been added which recite the use of a single restriction endonuclease. They are supported at page 8, first paragraph, of the specification which teaches:

> One can amplify without contamination or with much reduced contamination by digesting all of the reagents for amplification with **a restriction endonuclease**. *Alu* I is a preferred enzyme although others can also be used. The considerations for selecting **an endonuclease** are that it should not recognize and cleave within the primer sequences, but it should cleave at least once, twice, or thrice within the amplicon.

(emphasis added). It is respectfully submitted that no new matter is added by this amendment.

The Rejection of Claims 2, 4, 8-10, 15, and 18 Under 35 U.S.C. § 103(a)

Claims 2, 4, 8-10, 15, and 18 have been rejected under 35 U.S.C. § 103(a) as unpatentable over Steinman (U.S. 5,516,292) in view of DeFilippes (*Biotechniques 10*:26-30(1991)). Applicants respectfully traverse the rejection.

Claim 2, the only independent claim of the rejected claim set and from which all other rejected claims depend, is directed to a method of performing *in vitro* amplification that includes a step of digesting reagents for *in vitro* amplification with *Alu*I restriction endonuclease. The method further comprises steps of inactivating the *Alu* I, mixing in a test sample, subjecting the mixture to amplification conditions, and detecting any amplification products formed.

To reject claims as *prima facie* obvious the Patent Office must meet three criteria:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or

> references when combined) must teach or suggest all the claim limitations.

MPEP § 2143. The *prima facie* case of obviousness of claims 2, 4, 8-10, 15, and 18 must fail because one of ordinary skill in the art would not have been motivated to combine the teachings of Steinman and DeFilippes to arrive at the claimed invention, *i.e.*, the *prima facie* case fails to meet the first criterion.

When determining the patentability of a claimed invention which combines two known elements, there must be something in the prior art as a whole to suggest the desirability, and thus the obviousness, of making the combination. *In re Beattie*, 974 F.2d 1309 (Fed. Cir. 1992) (quoting *Lindemann*, 730 F.2d at 1462 (Fed. Cir. 1984)). As a prior art reference must be considered in its entirety, even portions that would lead away from the claimed invention must be considered. When there is such a clear teaching away in the prior art, the prior art would not suggest the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540 (Fed. Cir. 1983). It is improper to combine references where the references teach away from their combination. *In re Grasselli,* 713 F.2d, 731, 743, 218 USPQ 769, 779 (Fed.Cir.1983). "[T]he question is whether there is something in the prior art as a whole to suggest the desirability, and thus the obviousness, of making the combination," not whether there is something in the prior art as a whole to suggest that the combination is the most desirable combination available. *In re Fulton,* 391 F.3d 1195, 1200 (Fed. Cir. 2004), quoting *In re Beattie*, *supra*. But, a reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference or would be led in a direction divergent form the path that was taken by the applicant. *In re Gurley,* 27 F.3d 551, 553 (Fed. Cir. 1994). Under the proper legal standard, a reference will teach away

when it suggests that the developments flowing from its disclosures are unlikely to produce the objective of the applicant's invention. *Id.*

Steinman is cited as teaching all of the elements of the method of independent claim 2 except the use of Alu I restriction endonuclease. As recognized in the Office Action, Steinman does not teach digesting reagents for *in vitro* amplification with *Alu*I restriction endonuclease. DeFilippes is cited as teaching a different method for decontamination in which AluI restriction enzyme is used but the decontamination step does not include Taq DNA polymerase.

DeFilippes tested *Alu*I for its ability to successfully decontaminate *in vitro* amplification reagents. However, DeFelippes found that *Alu* I was not suitable for this purpose. De Felippes found that "digestion with *Alu*I, illustrated in Figure 1, **was not routinely successful**, and in some cases a light band was present at the proper position in lane 7 although no template was added after the inactivation of the *Alu*I." Page 28, sentence spanning columns 2 and 3. Using the same test template but a smaller amplification target, DeFelippes reported, "In this case also, *Alu*I **did not always completely inactivate** the template." Page 28, column 3, lines 9-11.

When DeFilippes is considered as a whole, DeFilippes teaches away from the use of AluI in such an *in vitro* amplification method because DeFilippes teaches that *Alu*I does not effectively digest *in vitro* amplification reagent mixtures, *i.e.*, *Alu*I does not effectively eliminate contaminating DNA that is present in reagents for *in vitro* amplification. DeFilippes teaches that in both a first and a second set of test assays, *Alu*I did not successfully decontaminate the reagents for *in vitro* amplification.

It is respectfully submitted that the PTO's assessment of DeFelippes is based on a faulty interpretation of the implications of DeFilippes' explicit teachings. While DeFilippes may appear on first reading to teach that *Alu*I was operative for decontamination, albeit not

completely operative, such an interpretation ignores the inherent characteristics of *in vitro* amplification and the uses to which *in vitro* amplification are applied. *In vitro* amplification, by its very design, amplifies extremely small quantities of DNA, even single molecules. Once amplification begins, the reaction is exponential. When *in vitro* amplification is used to detect very rare templates in a sample, it is often performed for many cycles, beyond the "exponential phase" of the reaction to ensure detection of rare events. This practice makes it impossible to distinguish between templates that are present as rare members of a sample population and members which are prevalent in a sample. Moreover, low level contaminants are indistinguishable from rare analytes even if *in vitro* amplification is limited to low amplification cycle numbers.

The art has long recognized that effective decontamination of *in vitro* amplification reactions requires ***complete*** elimination of contaminating DNA. Anything less leads to false positive results due to the extreme sensitivity of the reaction.

- "The very feature that makes nucleic acid amplification systems so powerful, that is, their high degree of sensitivity, also makes them prone to false-positive results because of inadvertent contamination by nucleic acids." Rys *et al., J. Clin. Microbiol. 31:* 2356, 1993. Exhibit A
- "Because of the extremely sensitive nature of the PCR process, contamination from carrying over of previously amplified PCR products in the same laboratory can be a serious problem. This problem affects both the diagnostic and the quantitative PCR and may be the most formidable problem in PCR application today." Ma, Chest Journal, 108:1393, 1396, 1995. Exhibit B

- "Unfortunately the PCR's exquisite capacity for amplification was accompanied by its extreme sensitivity to the presence of its own product as a feedback contaminant. Because of the need for some molecular diagnostic laboratories to routinely detect less than 100 copies of certain target templates (e.g., viruses), this susceptibility of the PCR to trace amounts of its own product means the laboratory has a continual requirement to control a species it cannot easily detect, see or readily remove." Mifflin, "Control of Contamination Associated with PCR and Other Amplification Reactions," Molecular BioProducts, 1997. http://www.mbpinc.com/html/pdf/techreport/MifflinReport.pdf , Exhibit C
- "However, because an assay such as PCR is extraordinarily sensitive analytically, the slightest exogenous contamination with previously amplified HIV-1 DNA (carryover) causes a false-positive test result in an assay that is 'very highly specific' (analytically). The result, therefore, may be that analytical and diagnostic specificity diverge because the assay maintains its very high analytical specificity but becomes diagnostically misleading because of external contamination." Saah, *et al., Annals of Internal Medicine 126:*91-94, 1997. See page 3 of full-text version. Exhibit D
- "The exquisite sensitivity of the polymerase chain reaction means DNA contamination can ruin an entire experiment." Kok *et al., Nature 18:*339 1989. Exhibit E
- "The impressive sensitivity is, however, the greatest pitfall of the technique, as just a few contaminating DNA fragments can initiate a false positive result."

Lisby, *Ugeskr Laeger.* 155:1708, 1993. Exhibit F

- "However, due to its extreme sensitivity, the PCR is highly susceptible to contamination, resulting in false positive results." Krone, et al., *J. Acquir. Immune Defic. Syndr. 3:*517, 1990. Exhibit G
- "The sensitivity of the polymerase chain reaction (IPCR) can mean that even very low levels of contamination with the target DNA will result in a positive signal. At present this aspect is a major limitation in the use of PCR as a routine diagnostic method." Fox *et al., J. Virol. Methods. 33:*375, 1991. Exhibit H
- "In the very beginning of polymerase chain reaction (PCR) tests entering the field of diagnosis of infectious agents, the introduction of this technology into routine diagnosis was hampered by its frequent tendency to create false-positive results because of contamination." Burkardt, *Clin. Chem. Lab. Med. 38:*87, 2000. Exhibit I
- "Potential limitations of the technique for diagnostic purposes include mainly the possibility of false-positive results due to contamination...." Coutlee *et al., Mol. Cell Probes. 5:*241, 1991. Exhibit J

Thus, the art clearly recognized that even small numbers of contaminating templates ("just a few contaminating fragments") can cause the assay to fail. Thus, when DeFilippes taught that *Alu*I treatment yielded a light band on a gel or did not completely inactivate the contaminant template, he taught that *Alu*I was not suitable for the intended purpose. An enzyme that incompletely inactivates contaminant templates is simply not suitable for an amplification reaction. An amplification reaction must eliminate all contaminant, because **any** level of

contaminant can be confused with a rare analyte. Moreover, in the context of a diagnostic assay where one determines the presence or absence of an analyte, contamination will cause the assay to yield false positive results. False positive results are very important in the context of diagnosis, causing treatment where none is necessary, or causing the wrong treatment. Claims 5, 6, 11, 12, 13, 14 involve clinical samples which are used for diagnostic assays.

The PTO has taken the position that DeFilippes does not, in fact, constitute a teaching away from the invention, but merely describes *Alu*I as somewhat inferior to some other product for the same use. The PTO concludes, "In the instant context, DeFilippes teaches the effectiveness of Alu I digestion of *in vitro* amplification reagents in eliminating contaminated DNA...." On that basis, the PTO concludes that DeFilippes would have motivated one skilled in the art to modify the method of Steinman by using *Alu*I.

However, as demonstrated above, those of ordinary skill in the art recognize that a decontamination method that leaves any contamination is a problem in a technique that is as sensitive as *in vitro* amplification is. The art further recognized that this is especially true in the diagnostic context. Thus one of ordinary skill in the art would consider that DeFilippes does not suggest the desirability of combining *Alu*I with the method of Steinman. To the contrary, DeFilippes teaches that the use of *Alu*I is unlikely to produce the objective of the applicant's invention, because it does not successfully or routinely remove all contamination. Thus, one of ordinary skill would have been discouraged from following the path of DeFilippes and would have been led in a direction divergent from that path.

One of ordinary skill in the art would not have been motivated to employ AluI as the restriction endonuclease as taught by DeFilippes in Steinman's method of digesting *in vitro*

amplification reagents with a restriction endonuclease. DeFilippes teaches away from using *Alu*I to digest *in vitro* amplification reagents because DeFilippes teaches that *Alu*I cannot be relied upon to cleave the DNA that contaminates *in vitro* amplification mixtures. Thus, the *prima facie* case of obvious of claims 2, 4, 8-10, 15 and 18 must fail.

Applicant respectfully requests withdrawal of this rejection.

The Rejection of Claims 5-7, 11-14, 16-17, and 19-22 Under 35 U.S.C. § 103(a)

Claims 5-7, 11-14, 16-17, and 19-22 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Steinman in view of DeFelippes and further in view of Hoshina *et al.* (U.S. Patent No. 5,571,674).

Claims 5-7, 11-14, 16-17, and 19-22 depend from claim 2. Claim 2 recites a method of performing *in vitro* amplification which comprises a step of "digesting reagents for polymerase chain reaction with *Alu*I restriction endonuclease." Claim 5 recites the sample is a treated blood sample. Claim 6 recites that the blood sample is from a patient suspected of systemic bacteremia. Claim 7 recites that the primers comprise sequences as shown in SEQ ID NO:1 and SEQ ID NO:2. Claim 11 recites that the blood sample was treated to extract DNA therefrom. Claims 12 and 13 recite that the sample is urine and cerebrospinal fluid, respectively. Claim 14 recites that the primers hybridize to at least 10 eubacterial species' DNA in regions which are highly conserved. Claims 16, and 17-19 each recite a further step of the method of claim 2 for identifying a bacterial species as a source of the templates. Claim 20 recites that the Taq DNA polymerase is not active under the conditions used for the step of digesting. Claims 21 and 22 recite that the amplified product comprises at least 1 or 2 recognition sites for the *Alu*I restriction endonuclease, respectively. Steinman and DeFelippes are applied as above with regard to

independent claim 2. Hoshina is cited as teaching the recitations of each of these dependent claims.

To reject claims as *prima facie* obvious the Patent Office must meet three criteria:

> First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations.

MPEP § 2143. The *prima facie* case of obviousness of claims 5-7, 11-14, 16-17, and 19-22 must fail for the same reason discussed above with respect to independent claim 2: one of ordinary skill in the art would not have combined the teachings of Steinman and DeFilippes to arrive at the invention of independent claim 2. Thus the *prima facie* case fails to meet the first criterion.

Steinman teaches a method of performing PCR. Steinman, however, does not teach or suggest digesting reagents for PCR with *Alu*I restriction endonuclease. The Office Action acknowledges that Steinman fails to teach or suggest digesting reagents for PCR with *Alu*I restriction endonuclease. Office Action at page 5, line 9.

Hoshina, like Steinman, fails to teach or suggest digesting reagents for PCR with *Alu*I restriction endonuclease. In fact, Hoshina does not teach restriction endonuclease digesting reagents for PCR. As demonstrated above, one of ordinary skill in the art would not have been motivated to combine *Alu*I with the teachings of Steinman because DeFelippes taught that *Alu*I was ineffective in completely decontaminating PCR reagent mixtures. This teaching would have led those of ordinary skill in the art away from the invention because the teaching would clearly indicate that *Alu*I was not good for the intended purpose, *i.e.*, complete decontamination. Thus DeFelippes would not have motivated one of ordinary skill in the art to make the claimed

combination.

Thus, the combination of Steinman, DeFelippes, and Hoshina fails to teach or suggest "digesting reagents for polymerase chain reaction with *Alu*I restriction endonuclease" as recited in claim 2 and its dependent claims 5-7, 11-14, 16-17, and 19-22. The combination of Steinman, DeFelippes, and Hoshina thus fails to render the claimed invention obvious. The *prima facie* case of obviousness of these claims must fail.

Applicant respectfully requests withdrawal of this rejection.

Respectfully submitted,

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Preventing False Positives: Quantitative Evaluation of Three Protocols for Inactivation of Polymerase Chain Reaction Amplification Products

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False-positive results because of carryover contamination by previously amplified nucleic acids are currently the greatest impediment to routine implementation of nucleic acid amplification protocols. We evaluated three methods for inactivation of a 156-bp *Borrelia burgdorferi* polymerase chain reaction (PCR) product: (i) post-PCR cross-linking with isopsoralen (IP), (ii) pre-PCR treatment of a dU-containing PCR product with uracil *N*-glycosylase (UNG), and (iii) post-PCR alkaline hydrolysis (primer hydrolysis) of PCR products synthesized by using primers containing 3′ ribose residues. The sensitivities of the PCR performed under the conditions of each protocol were comparable. Inactivation of amplified DNA was highly efficient for all three protocols; the IP and UNG protocols eliminated at least to 3×10^9 copies of the product. The primer hydrolysis protocol varied in efficiency depending on the number and position of the 3′ ribose residues, but inactivation ranged from 10^4 to 10^9 copies. We conclude that with some modifications, all three systems are effective for eliminating amplified DNA products. Routine implementation of at least one method should help to avoid false-positive results because of carryover contamination.

The very feature that makes nucleic acid amplification systems so powerful, that is, their high degree of sensitivity, also makes them prone to false-positive results because of inadvertent contamination by nucleic acids. Such contamination occurs primarily from the following three sources: (i) cloned target molecules in plasmid vectors that are initially used for isolation and characterization of the target sequence; (ii) DNA carried over from clinical specimens containing large numbers of organisms, from cultures used to grow the organism, or within reagents used for amplification (17, 22); and (iii) the products of the amplification reactions themselves (12–15). The first two types of contamination can be avoided by avoiding areas in which cloning and sequencing of target DNA has been carried out and by using careful laboratory technique. However, contamination by previously amplified nucleic acids may occur in any laboratory and is thus the most likely form of contamination to be encountered in the course of routine testing. Although careful laboratory technique, including physical separation of pre- and postamplification steps (14), can delay the onset of contamination, such problems still occur and may be extremely difficult to overcome.

The ultimate long-term success or failure of all enzyme-catalyzed nucleic acid amplification methods likely depends on how well the products of amplification are contained or inactivated. Fortunately, in the last few years, inactivation protocols that use chemical, photochemical, and enzymatic methods for reducing the problems associated with amplicon contamination have been developed (1–11, 16, 18–21, 23, 25–27). It is presumed that the routine implementation of one or more of these protocols, coupled with careful laboratory technique and strict quality control procedures, will help to avoid carryover contamination in the clinical laboratory setting. However, no independent studies comparing the effectiveness of these methods have been published to date. In the study described here, we compared three different protocols for inactivation of known numbers of a *Borrelia burgdorferi* amplification product (24), and in this report we comment on the efficiency and usefulness of these systems for preventing false-positive polymerase chain reaction (PCR) results.

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MATERIALS AND METHODS

Accurate quantitation of PCR products by determining the A_{260} of gel-purified DNA is extremely difficult because of interference from even small quantities of oligonucleotides and deoxyribonucleoside triphosphates (5, 11). Thus, in order to standardize the DNA input amounts for each inactivation protocol, we performed quantitative dot blot hybridization. A dot blot was prepared on a Hybond N nylon membrane (Amersham Corp.) by using a Minifold I Microsample Filtration Manifold (Schleicher & Schuell). Tenfold serial dilutions of amplicon obtained by each method were prepared. A total of 5 μl of each dilution was added to 195 μl of cold TE (10 mM Tris-HCl, 1 mM EDTA), 20 μl of 3 M NaOH was added, and samples were incubated at 65°C for 60 min to denature the DNA. Tubes were cooled on ice, briefly centrifuged to remove condensation, 180 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was added, and the samples were stored on ice until they were blotted. Samples were blotted and probed with a ^{32}P-end-labeled probe as described previously (24). A dilution of a known concentration of a plasmid DNA (p197-OspAB-N40) containing the target sequence was used to estimate the copy number of the various amplified DNA products. The radioactivity in each well was quantitated by using an AMBIS scanner (AMBIS, Inc.) after hybridization. Beta emissions, measured in counts per minute, were obtained to determine the relative dilution factors required for standardization of amplicon concentrations.

A standard PCR master mixture was modified as appropriate for each inactivation protocol as follows: 1× PCR buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.75 mM $MgCl_2$, 0.01% bovine serum albumin); 200 μM (each) dATP, dCTP, dGTP, and dTTP (200 μM dUTP substituted for dTTP in the uracil *N*-glycosylase [UNG] protocol only); 50 pmol of oligonucleotide primers OSPA2 and OSPA4 (the corresponding 3'-ribo primer pairs were substituted as described [Integrated DNA Technologies, Inc., Coralville, Iowa]); 10% glycerol; 1.25 U of AmpliTaq; 100 μg of isopsoralen compound 10 (IP-10) per ml in the IP protocol only, and 0.5 U of UNG (Perkin-Elmer Corp.) in the UNG protocol only. Water was added to yield a 50-μl reaction volume. Target input (5 μl per 50-μl reaction) consisted of the plasmid dilution, the treated or untreated amplicon dilution, or water for the negative controls.

Unmodified primer sequences were as follows: OSPA2, GTT TTG TAA TTT CAA CTG CTG ACC; OSPA4, CTG CAG CTT GGA ATT CAG GCA CTT C; OSPA3 (probe), GCC ATT TGA GTC GTA TTG TTG TAC TG.

All reactions were performed in a DNA thermal cycler (model 480; Perkin-Elmer Corp.). The same thermal cycler profile was used in all experiments to facilitate comparison among methods. The components were incubated at room temperature for 10 min and were denatured at 95°C for 10 min prior to 45 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min; this was followed by a final extension at 72°C for 7 min and a 72°C hold at the completion of the profile. The initial incubation at room temperature and the incubation at 95°C were included to accommodate the UNG protocol and were adopted in all subsequent experiments to control for the possible loss of AmpliTaq enzyme activity. A minimum annealing temperature of 55°C was used since UNG has enzymatic activity below 55°C (27), which may decrease amplicon yield. A 72°C hold file prevents possible degradation of the amplicon prior to analysis because of residual UNG activity. Reactions were cooled rapidly to 4°C before removal from the thermal cycler.

In order to determine the inactivation efficiency of each protocol, 10-fold dilutions of the quantitated amplification reactions were prepared as described above and 5-μl aliquots from parallel dilution series of treated and untreated amplicons were reamplified by using the conditions described. The inactivation efficiency was determined by comparing the number of positive reamplification reactions in each series prepared from treated reactions relative to those of a parallel control series prepared from untreated amplification products. The last positive PCR in a dilution series defined the inactivation efficiency.

The 156-bp products were visualized on ethidium bromide-stained 3% NuSieve–1% SeaKem agarose gels (FMC Bioproducts). Amplification products were denatured in the gel and were transferred to a nylon membrane (Hybond N; Amersham Corp.) by Southern blotting as described previously (24), except that the membranes were cross-linked by using a Hoefer UV cross-linker. The ^{32}P-end-labeled OSPA3 internal oligonucleotide probe was prepared as described previously (24), and hybridizations were performed in glass bottles in a Hybaid Mini Hybridization Oven (National Labnet). After 1 h of prehybridization in 10 ml of hybridization solution (5× Denhardt's solution, 5× SSPE [20× SSPE is 2.98 M NaCl, 0.2 M $NaPO_4$, and 0.02 M EDTA; pH 7.4], 0.5% sodium dodecyl sulfate [SDS], 100 μg of denatured salmon sperm DNA per ml), the labeled probe was added (180 ng per hybridization) and hybridized for 3 h at 55°C. Filters were washed twice for 10 min with 150 ml of wash buffer 1 (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% SDS) and once for 30 min at 55°C with 150 ml of prewarmed wash buffer 2 (1× SSC, 0.1% SDS). Autoradiograms were prepared by exposing Kodak X-Omat film overnight to 48 h at −70°C.

IP protocol. Isopsoralen inactivation was performed essentially as described by Cimino et al. (5) and Issacs et al. (11). A volume of untreated amplicon containing 1.2×10^{10} copies of the 156-bp product was divided into four 50-μl aliquots. An equal volume of water or IP-10 (final concentration, 100 μg/ml; HRI Associates) was added when appropriate, resulting in the following four test samples (3×10^9 copies in each): I (−IP, −UV), II (−IP, +UV), III (+IP, −UV), IV (+IP, +UV). Samples II and IV were exposed to UV light (365 nm) for 15 min at 4°C in an HRI 100 UV Photochemical Reaction Chamber (HRI Associates). After treatment, 10-fold dilution series were prepared as described above and 5-μl aliquots were reamplified and detected after dot blotting or Southern blotting.

UNG protocol. The UNG protocol functions by incorporating dUTP into amplification products, which are selectively degraded by UNG (16, 20). A standardized dU-containing amplicon preparation was prepared by amplification of template DNA in the presence of dATP, dCTP, dGTP, and dUTP. The quantity of the dUTP-containing product was then determined by quantitative hybridization and was adjusted to a final concentration of 3×10^{10} copies per 50-μl reaction mixture. A 10-fold dilution series was made starting from 5 μl (3×10^9 copies), and two sets of reamplification reactions, with and without UNG (Perkin-Elmer Corp.), were prepared. After amplification, the products were dot blotted and detected as described above.

PH protocol. The primer hydrolysis (PH) protocol was performed essentially as described in the package insert (Integrated DNA Technologies, Inc.). Modified primers containing one or two ribose residues at or near the 3' end were synthesized. The 3'-ribo primer sequences (kindly provided by Integrated DNA Technologies, Inc.) were as follows: OSPA2r1, GTT TTG TAA TTT CAA CTG CTG ACr(C); OSPA4r1, CTG CAG CTT GGA ATT CAG GCA CTTr(C) (set A); OSPA2r2, GTT TTG TAA TTT CAA CTG CTG Ar(C)r(C); OSPA4r2, CTG CAG CTT GGA ATT CAG GCA CTr(U)r(C) (set B); OSPA2r2 spaced, GTT TTG TAA TTT CAA CTG CTG r(A)Cr(C); OSPA4r2 spaced, CTG CAG CTT GGA ATT CAG GCA Cr(U)TrC (set C) (r indicates ribose linkage). Amplicon preparations were made according to the conditions described above. After amplification, 100-μl aliquots were treated with the reagents supplied by the manufacturer, as follows. A total of 25 μl of solution 1 (5 M NaOH) (for hydrolysis) was added and tubes were incubated for 30 min at 95°C in a thermal cycler and cooled to room temperature (time delay file linked to soak file), and then 25 μl of solution 2 (5 M HCl) (for neutralization) was added. Untreated 100-μl samples were processed in parallel, substituting water for the NaOH and HCl solutions. Tenfold dilution series were prepared from treated and untreated samples, and 5-μl aliquots of each dilution were subjected to reamplification and detection of resulting products.

RESULTS

Effects of inactivation on product detection. The photocross-linking of DNA by isopsoralens results in covalent modification of one strand of the DNA molecule. We hypothesized that this covalent modification would have an

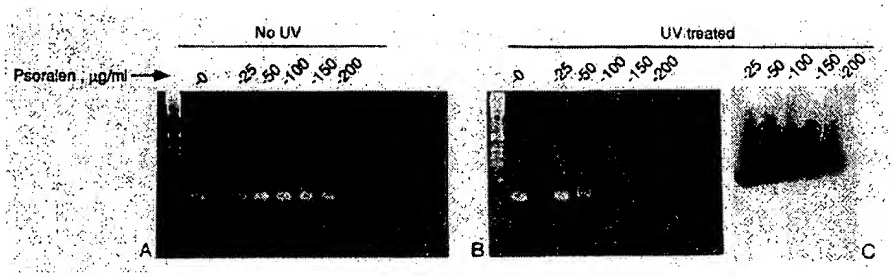

FIG. 1. Effects of IP inactivation on gel mobility and hybridization of PCR products. Concentrations of IP-10 (HRI Associates) ranging from 0 to 200 μg/ml were added to PCR mixtures prior to amplification. Without UV treatment (A) there was a slight dose-dependent decrease in amplification efficiency at 200 μg of IP per ml. After UV exposure (B and C), there was a concentration-dependent increase in the apparent molecular mass of the PCR product and a decrease in ethidium bromide staining intensity. A slight dose-dependent decrease in hybridization of the internal oligonucleotide probe is also observed at the highest IP-10 concentrations (100 μg of IP-10 per ml was selected for further inactivation experiments).

effect on the electrophoretic mobilities of the PCR products or their staining by ethidium bromide. Accordingly, for the IP protocol, we observed a stepwise increase in the apparent molecular mass of the amplification products and a concomitant decrease in ethidium bromide staining intensity that was directly dependent on the IP concentration (Fig. 1A and B). However, this did not result in a significant loss of sensitivity after hybridization with an internal oligonucleotide probe except at the highest IP concentrations (Fig. 1C). Consequently, we chose 100 μg of the IP compound per ml for further inactivation experiments. Amplification products that were prepared according to the conditions of the PH and UNG protocols had no apparent effect on electrophoretic mobility, ethidium bromide staining, or hybridization efficiency (data not shown).

Effects of the inactivation protocols on product yield and reaction sensitivity. Two of the inactivation protocols required modification of conditions in order to overcome decreases in reaction sensitivity. For the IP protocol, to counteract a slight inhibitory effect of the IP on PCR at high IP concentrations, 10% glycerol was added to the reactions. This resulted in sensitivity that was equal to that of the unmodified reactions (Fig. 2A, top two rows). For the UNG protocol, a substantial loss of reaction sensitivity was initially observed when the enzyme was added to the reaction mixture prior to amplification because of the breakdown of amplification products via residual UNG activity during or shortly after amplification (data not shown) (27). In order to overcome the latter problem, reactions were held at 72 or 4°C (instead of room temperature) prior to analysis. The PH method required no alteration of existing protocols except for substitution of the modified primers.

With appropriate modifications in place, the sensitivities of the amplification reactions and the apparent yield of amplification products obtained for each protocol were comparable; for the IP, UNG, and PH protocols, untreated dilution series produced detectable amplification products in the 10^{-8} dilutions (Fig. 2A and B; Fig. 3). Reactions in the 10^{-9} dilution were observed in many experiments, which was consistent with the Poisson distribution of single-molecule detection by PCR (Fig. 2 and 3).

Efficiency of inactivation of previously amplified DNA. For inactivation of the 156-bp *B. burgdorferi*-specific amplification product, all three protocols were of comparable efficiency. The IP and UNG protocols resulted in inactivation of all input copies (corresponding to ca. 3×10^9 copies) of the amplification product (Fig. 2). Interestingly, whereas UV light alone seemed to produce some inactivation of the amplified products (Fig. 2A, second row from the bottom), inactivation was incomplete; at least low levels of active template were detected in all but the last dilution. The combination of UV light plus IP treatment eliminated contaminating templates up to the limit of the study (3×10^9 copies). Preincubation of dU-containing templates with UNG also resulted in elimination of reamplifiable templates at all of the tested concentrations (Fig. 2B).

For the PH protocol, the efficiency of inactivation varied with the primers used for amplification. The primers containing single 3′ ribose linkages yielded a product that was completely inactivated at all of the tested concentrations by base hydrolysis (Fig. 3A). Unexpectedly, the primers containing two ribose linkages yielded incompletely inactivated products (Figs. 3B and C). For these latter primers, inactivation of only 10^4 and 10^5 copies was observed following base hydrolysis. This incomplete inactivation may have been due to the loss of the ribose residues during primer synthesis or storage, resulting in products that were resistant to alkaline hydrolysis.

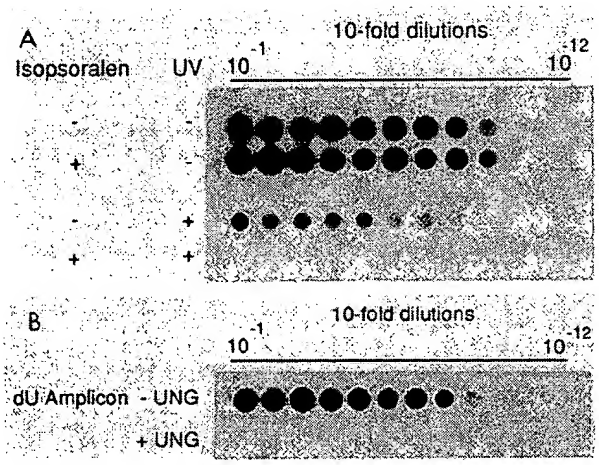

FIG. 2. Inactivation efficiency by the IP and UNG protocols. (A) Inactivation by isopsoralen. Tenfold serial dilutions of the amplified products were reamplified after treatment according to the conditions indicated on the left. (B) Inactivation by UNG. A 10-fold dilution series of dU-containing product was reamplified with (bottom row) or without (top row) UNG pretreatment.

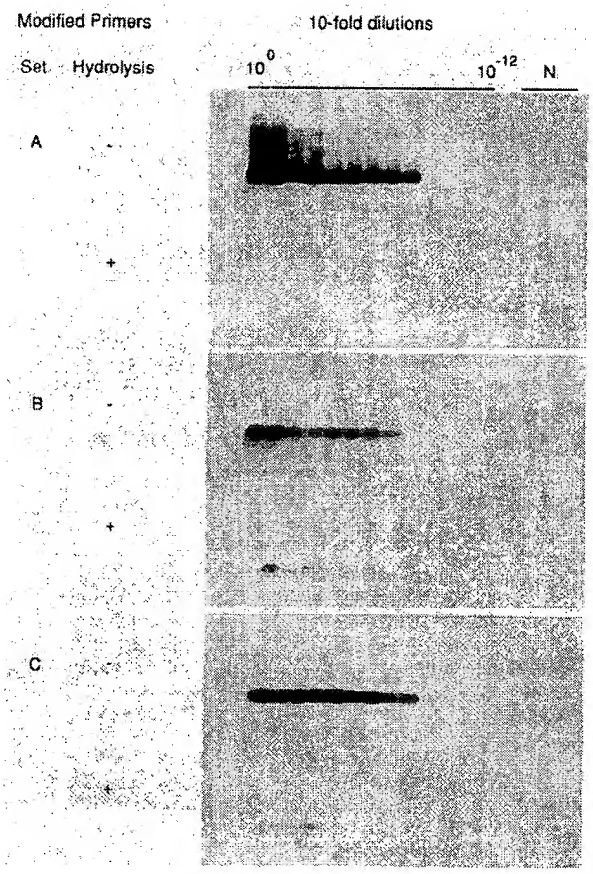

FIG. 3. Inactivation by primer hydrolysis. PCR products were generated by amplification with 3'-ribo primer set A (A), set B (B), and set C (C). A 10-fold dilution series was made from products with and without alkaline posttreatment. Untreated reamplification series are shown at the top of each panel.

DISCUSSION

Despite the description in the literature and the commercial availability of reagents and procedures for the control of PCR product contamination (5, 10, 11, 20), these methods have infrequently been used in published PCR applications to date. This may be due in part to the lack of formal evaluations of these methods by an independent laboratory. In the present study we challenged three such protocols with the same amplification product under conditions that were determined to minimize artifactual differences in inactivation efficiency (i.e., introduction of unmodified templates into the UNG protocol). The photochemical procedure (IP protocol) incorporates a photochemically reactive isopsoralen into the amplification mixtures prior to amplification. After amplification, but before the reaction tubes are opened, the tubes are exposed to UV light, which produces photochemically induced single-stranded DNA adducts. Once this reaction occurs, the likelihood of reamplifying a contaminating template is greatly reduced because DNA polymerases are unable to extend past the photochemical modifications of the template. The enzymatic method (UNG protocol) exploits the ability of UNG to selectively degrade DNA templates containing deoxyuracil. If routine PCR amplification is carried out by using deoxyuracil triphosphates in place of thymidine triphosphates, laboratory-generated dU-containing contaminants are degraded prior to amplification by a brief incubation of the reaction mixtures in the presence of UNG. The third method (PH protocol) uses modified primers containing one or two ribose residues near their 3' ends. After amplification, the tubes are opened and NaOH is added. The ribose linkages (but not the DNA linkages) are susceptible to base hydrolysis, and a cleavage that greatly reduces the efficiency of reamplification of contaminating templates is produced in each strand (28).

In theory, all enzyme-catalyzed nucleic acid amplification methods are susceptible to false-positive reactions because of amplified substrate accumulation. A typical PCR amplification reaction may contain up to 10^{12} copies of an amplified template; the inactivation protocols evaluated in the present study are thus capable of eliminating all but a few hundred copies of a contaminating template. The IP protocol theoretically inactivates the native template in addition to the amplified product; thus, a net loss in the number of active templates may actually be possible, since many reactions will contain more than 100 copies of the target nucleic acid at the start. This may become an important consideration for clinical laboratories that perform amplifications with clinical samples containing thousands or even millions of copies of the target organism, as is the case for bacterial cultures and for viruses such as hepatitis B virus.

The effectiveness of the IP and UNG inactivation protocols is theoretically influenced by the percentage of G+C residues present in the product and by the length of the product (5, 11). In our study, both protocols were challenged with a relatively short (156-bp) product, but they were nonetheless effective. This is probably a function of the low G+C content of the product tested. The photochemical reactivities of isopsoralens are enhanced for DNA sequences rich in A+T residues, since these compounds are more likely to form cross-links with A+T-rich regions of the helix. Likewise, the UNG protocol is probably more effective on products with low G+C contents because of the high concentration of deoxyuridine target residues within the product. In the accompanying manuscript (5a), the efficiencies of the IP and UNG protocols are shown to vary widely depending on the length and G+C content of the product. Thus, the choice of a system for inactivation of a PCR product must take into consideration the size of the product and its nucleic acid composition. The conditions for using a protocol should be established and evaluated for each target system.

Each of the systems that we evaluated in the present study presented certain drawbacks. The IP protocol uses potentially hazardous isopsoralen compounds that, like ethidium bromide, must be handled with care in the laboratory. Although the reagent itself is inexpensive, a dedicated UV transilluminator must be purchased for maximum effectiveness. The UNG protocol is expensive because of the higher cost of dUTP compared with that of TTP and the requirement for an extra enzyme in the reaction mixtures. Furthermore, care must be taken to prevent residual UNG activity from destroying the amplification product prior to analysis. Our experience has been that most reactions tolerate substitution of dUTP for dTTP (5a), but some do not (20). Poor reaction efficiency after dUTP substitution is probably due to the lower incorporation efficiency of dUTP by *Taq* polymerase or to changes in primer annealing on dUTP-substituted templates. The primer hydrolysis method appears to be highly effective and is cost competitive, but as it is currently performed, tubes containing amplified DNA must be opened

to allow the addition of NaOH. Until reaction vessels that allow unidirectional reagent addition after amplification are developed, the opening of the tubes may provide opportunities for contamination by aerosolized amplification products.

None of the methods described here is mutually exclusive. Isopsoralens can be used to inactivate dU-containing DNA, and the amplifications themselves can be performed with ribose-modified primers. Indeed, our general approach to implementation of an inactivation protocol is to routinely replace dUTP for TTP in amplification mixtures, but to use IP inactivation as the frontline prophylactic measure. Thus, if contamination occurs despite the use of the IP protocol, UNG can be added on an interim basis to control the problem until the source of contamination is identified.

Contamination control in laboratories that regularly perform PCR should incorporate an integrated approach to the prevention of amplicon carryover. Strict separation of pre- and postamplification steps and topical application of 10% sodium hypochlorite (bleach) to working surfaces and to spilled amplified product should be implemented (25). Despite these precautions, however, false-positive reactions may still occur because of low levels of contaminating amplification products that are carried over on the skin, hair, and clothing of the laboratory workers within various laboratories (12). Careful consideration of contamination control in the design of an amplification protocol may provide the final "missing link" in the evolution of practical and useful tests based on nucleic acid amplification.

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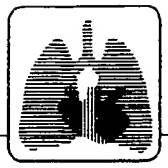

impact of basic research on tomorrow's medicine

Applications and Limitations of Polymerase Chain Reaction Amplification*

Tony S. Ma, MD, PhD

(CHEST 1995; 108:1393-1404)

bp=base pair; cDNA=complementary DNA; CSF=cerebrospinal fluid; DMD=Duchenne's muscular dystrophy; mRNA=messenger RNA; NSCLC=non-small cell lung cancer; PCR=polymerase chain reaction; Rep-PCR=PCR using the repetitive elements of bacterial genome as primers; RFLP=restriction fragment length polymorphism; rRNA=ribosomal RNA; RT=reverse transcription; RT-PCR=PCR amplification following RT; SCLC=small cell lung cancer

Key words: PCR; PCR application; PCR diagnosis; quantitative PCR

Principles of Polymerase Chain Reaction Amplification

In 1983, Dr. Kary Mullis from Cetus conceived of a novel concept.[1] By utilizing an enzyme that normally is responsible for DNA synthesis (a DNA polymerase), and two short pieces of laboratory-synthesized DNA of specific sequences (DNA primers), he invented a test-tube process of repetitive DNA synthesis.[2,3] This process is termed "polymerase chain reaction (PCR) amplification." This PCR amplification process in essence duplicates in a test tube the process of DNA synthesis utilized by all living organisms. The invention of PCR has lifted recombinant DNA technology to a new level: it allows the scientist and clinician to directly amplify a defined stretch of DNA sequence in an exponential fashion, in the short span of several hours. The simplicity and sensitivity of this process makes it possible to utilize this technology for the analysis of a minute amount of specific genetic material. Application of this powerful technology has touched every aspect of medical science and the practice of medicine, from the study of gene regulation to the diagnosis of infectious, neoplastic, and hereditary diseases. The invention of this technology ultimately earned Dr. Mullis the Nobel Prize for Chemistry in 1993.

The purpose of this article is to examine the theory, application, and limitations of PCR technology in basic research and in clinical medicine.

PCR Amplifies DNA Molecules in an Exponential Fashion

The principles of PCR amplification are demonstrated in Figure 1. DNA is made from four nucleotides: adenine, guanine, thymidine, and cytosine. The DNA molecule normally is present inside the cell as a complex of two polynucleotide strands which, at the molecular level, complement each other so that adenine (a purine) on one strand always corresponds to thymidine (a pyrimidine) on the other strand, and guanine (a purine) on one strand always corresponds to cytosine (a pyrimidine) on the opposite strand. This complementation allows maximum hydrogen bond formation between the purine and pyrimidine bases and at the same time provides for the same spacing for each of the base pairs (bp) (always a purine, a fused 5- and 6-member rings, with a pyrimidine, a six-member ring). The two DNA strands are oriented in an antiparallel direction (designated 5′ to 3′ in orientation according to the linkage of the phosphodiester backbone) and twist around each other, creating a "double helix."

DNA polymerase is capable of synthesizing a second strand of DNA, always in a 5' to 3' orientation, using the four nucleotide triphosphates as substrates, one DNA strand as a template, and a short piece of complementary DNA as a primer. To initiate DNA synthesis in a test tube, the double-stranded DNA is first heated (denatured) to separate the two strands (Fig 1). It is then cooled, thus allowing the annealing of the primers (which are provided in an excess amount) to the complementary DNA fragments. If two specific DNA primers are provided that are complementary to the DNA sequences on the opposite strand of the parental DNA molecule, DNA synthesis mediated by the DNA polymerase will yield two double-stranded molecules from the parental DNA duplex (Fig 1). If this

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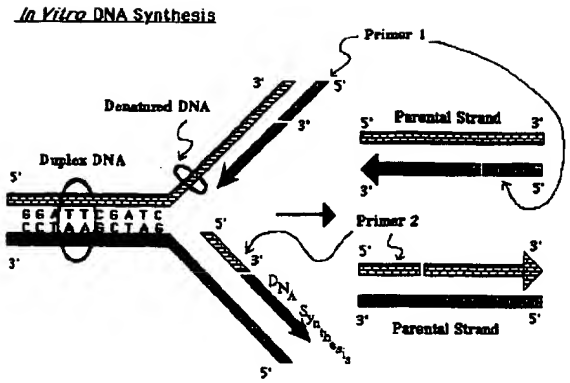

FIGURE 1. PCR amplification *In vitro* DNA synthesis. The two DNA molecule is oriented in an antiparallel direction (designated 5′ to 3′ in orientation according to the linkage of phosphodiester backbone). DNA polymerase can synthesize a second strand of DNA, always in a 5′ to 3′ orientation, using the four nucleotide triphosphates as substrate, one DNA molecule as a template, and a short piece of complementary DNA molecule as a primer. In a test tube, to initiate the DNA synthesis, the double-stranded DNA is first heated (denatured) to separate the two strands, thus allowing the annealing of the complementary primer. If two specific DNA primers are provided, primer 1 and primer 2, which are complementary to the opposite strand of the parent DNA molecule, this process would yield two double-stranded molecules from the one parental DNA duplex.

process is repeated a second time, the result would be four DNA molecules. In other words, the DNA multiplies exponentially (doubling each time) as the same cycle of reaction is repeated (Fig 2). This is the basis of PCR. PCR amplification theoretically can amplify a short stretch of DNA fragment (up to several thousand base-pairs long), from a single molecule to 1,000 copies (2^{10}=1,000) if the process is repeated 10 times, to 1 million copies (2^{20}=1,000,000) if the process is repeated 20 times, and to 1 billion copies (2^{30}= 1,000,000,000) if the process is repeated 30 times. This process can be carried out automatically in a thermal cycler that repeats this cycle of heat denaturation (94 to 96°C), annealing of primers (40 to 60°C), and DNA synthesis (65 to 75°C), using a thermal-stable DNA polymerase isolated from bacteria that live in thermal springs in Yellowstone National Park.[4] A billionfold amplification can be accomplished in the span of 3 to 4 h, yielding microgram quantity of DNA for direct analysis or further manipulation.

APPLICATIONS IN BASIC RESEARCH

Because of its power and ease in amplifying DNA sequences from minute sources, PCR has been applied to many areas of basic medical research. Three areas profoundly influenced by this invention are (1) molecular cloning, (2) message quantification, and (3) genetic linkage.

PCR in Molecular Cloning

Theoretically PCR should be able to detect a single molecule of DNA. This was initially demonstrated by experiments in which PCR amplification of serially diluted template material yielded products in accordance to an expected Poisson distribution consistent with a successful amplification of a single DNA molecule.[4] Subsequently successful amplification and analysis of DNA sequences using single sperm were reported.[5,6] The success of this endeavor is influenced by the purity of the DNA source and by the method for recovering the DNA. Nevertheless, single DNA molecule PCR has been shown to be both feasible and practical, and has been applied to investigations of human diseases. For example, it has recently been demonstrated with Reed-Sternberg cells picked from histologic sections of Hodgkin's lymphomas.[7] More commonly, PCR has been used as an adjunct in molecular cloning. The usefulness of this procedure as a primary cloning method is limited somewhat by the error rate of the Taq polymerase that has been estimated to be 10^{-4} per base pair (bp) per cycle of amplification.[4] This error rate is several times higher than the error rate of the Klenow enzyme and other DNA polymerases used in conventional cloning methods. PCR has been shown to be useful to recover a DNA fragment whose sequence has been previously characterized in the same or different species. The recovered DNA can be directly analyzed or can be used as a DNA probe for hybridization analysis (*eg*, Northern or Southern blot analysis, see below) or for further conventional cloning procedures. One of the most useful applications of PCR is for genetic analysis (see below), in which one or multiple regions of DNA from an individual is amplified for direct analysis. Another useful and rewarding approach is to use PCR to amplify novel or related genes. By analyzing several DNA sequences from members of a gene family or from homologous genes derived from different species, it is usually possible to identify short stretches of DNA where the DNA sequences are conserved through evolution or through speciation. DNA primers or degenerate primers (mixture of primers with two to four different nucleotide bases at positions of uncertainty) can then be synthesized based on the conserved sequences, and PCR amplification performed to recover gene fragments belonging to the same gene family. Using this approach, an ever-expanding number of complementary DNAs (cDNAs)—DNA complementary to a messenger RNA (mRNA)—have been cloned. It has recently been reported that lengths of DNA up to 35 kilobases can be amplified with high fidelity ($<10^{-5}$ mutations per bp per cycle of amplification) using a combination of two polymerases.[8] The general application of this new finding using more complex genomic DNA awaits further experimentation, but it is likely that, assisted by this and other PCR technologies, the list of cloned

cDNAs or genomic DNA for genes related to human diseases will expand broadly and rapidly.

PCR in DNA and RNA Quantification

The presence or the absence of a specific gene sequence is often insufficient to resolve a research or clinical question. For example, one may need to know the quantity or the concentration of a specific gene product: in the case of mRNA, this might correspond to a message level; in the case of a microorganism, this might be the total number of the infectious organisms. The level of mRNA and DNA can be semiquantitatively determined by Northern and Southern blot analysis, respectively, or by dot-blot analysis. In these approaches, purified RNA or DNA is bound to a supporting membrane (nylon or nitrocellulose), without (*ie*, dot blot) or with (*ie*, Northern or Southern blotting) an intervening electrophoresis step for size fractionation. The membrane with the immobilized nucleic acids is then incubated with a radiolabeled DNA or RNA fragment of known sequence (*ie*, probe). Following 12 to 16 h of incubation (termed hybridization), the probe binds to the complementary nucleotide sequence immobilized on the membrane. Excess probe is removed by washing with a low-salt buffer, and the retained probe that is specifically bound to the DNA or mRNA of interest is then visualized by exposing the membrane to x-ray film. The quantity of a specific message can be estimated by comparing the radioactive signal to that of a control sample. This process of measuring a specific DNA or RNA sequence is suboptimal for three reasons: (1) the quantitation is relative and interexperimental results cannot be easily compared; (2) the procedure requires substantial starting material and cannot be used for small samples such as those obtained from biopsy specimens, archival samples (*eg*, paraffin blocks), or clinical samples (such as blood or cerebrospinal fluid [CSF]) that might contain very low levels of specific cells or pathogenic organisms; and (3) the procedure is time consuming (requiring 3 to 4 days) and cannot be easily automated. Because PCR has been established to be extremely sensitive and easy to perform, the concept of "quantitative PCR" has evolved into current technologies, once it is appreciated that PCR amplification can be used to identify rare sequence from a complex mixture.[9] Quantitation has been attempted for DNA sequences, and for RNA sequences following reverse transcription (RT) step to generate cDNA (RT-PCR).

The major obstacle for quantitation using PCR, paradoxically, lies in its extreme sensitivity. As with the message to be amplified, the error of quantitation (*ie*, a varying efficiency of amplification during PCR) increases exponentially with each succeeding amplification cycle. Thus, the error can amplify exponentially in an unpredictable manner and theoretically invalidate experimental results. Two approaches to circumvent this problem have been proposed: (1) during reverse transcription of mRNA, a known quantity of synthesized mRNA is added, which can be differentiated from the endogenous message, and the two cDNAs are then amplified simultaneously in the same reaction medium using the same sets of primers;[10] (2) during PCR amplification, a known amount of modified DNA is added to the PCR medium to be used as competitor for the same DNA primers used for amplification of the endogenous target sequences.[11] In the first approach, the amplified unknown is compared with the amplified standards. In the second approach, the competitor competes with the unknown for the same primers. When the competitor is present at the same concentration as the unknown, both DNAs would amplify to the same final amount. Thus, by performing a series of PCR reactions each with a different amount of competing DNA, the endogenous DNA level can be deduced. Both approaches use multiple standards to determine one unknown. In addition, it should be noted that the "competitive PCR"[11] is done following the RT step; thus, the quantity obtained with competitive PCR does not directly reflect the mRNA level, as the efficiency of RT is not determined. A quantitative competitive PCR for RNA has been described[12] and used to quantitate HIV-1 RNA levels.[13] Competitive PCR has been advocated to be independent of the amplification cycle number and efficiency of amplification.[11] Theoretically, however, the amplification should be performed for cycles during which the exponential phase of growth for the DNA molecules occurs.[14,15]

For PCR, the amount of product N after n cycles of amplification is represented by $N=N_0(1+E)^n$, with E representing efficiency of amplification. If E=1 (100% efficiency), the equation becomes $N=N_o2^n$. The product would double with each subsequent cycle and the abundance of the original target sequence, N_o, can be precisely determined from extrapolation. To visualize this process, a diagram shown in Figure 3 is presented. In this figure the amount of DNA product in micrograms that is synthesized, starting from either 1, 10^2, 10^4, or 10^6 DNA molecules, is shown as a function of the number of PCR amplification cycles that is performed. Aerosolized DNA from previous PCR may contain 10^4 to 10^6 copies of DNA molecules (see below). One microgram of genomic DNA, for comparison, contains 3×10^5 copies of single-copy gene. Highly abundant mRNA such as creatine kinase M in the heart may be present at 10^{11} copies per 1 μg total RNA. Moderate abundant mRNA, such as creatine kinase B in the heart, may be present at 10^{10} copies per 1 μg total RNA.[16] As demonstrated in Figure 3, starting with 10^6 molecules, the PCR product increases exponentially for each cycle of amplification up until the 20th cycle,

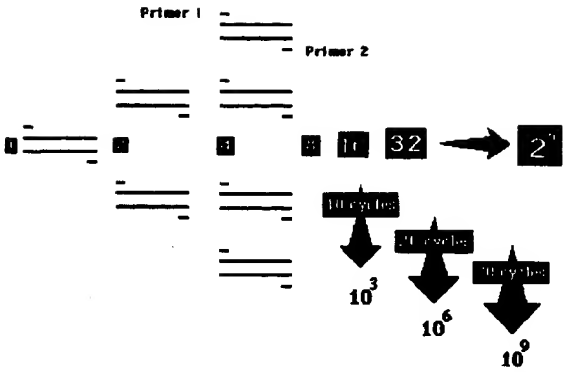

FIGURE 2. PCR amplification. Exponential amplification with PCR. The preceding process is repeated a second time, producing four DNA molecules. Starting with a single molecule, PCR amplification produces 1,000 copies of DNA (2^{10}=1,000) if the process is repeated 10 times, 1 million copies (2^{20}=1,000,000) if the process is repeated 20 times, and 1 billion copies (2^{30}=1,000,000,000) if the process is repeated 30 times.

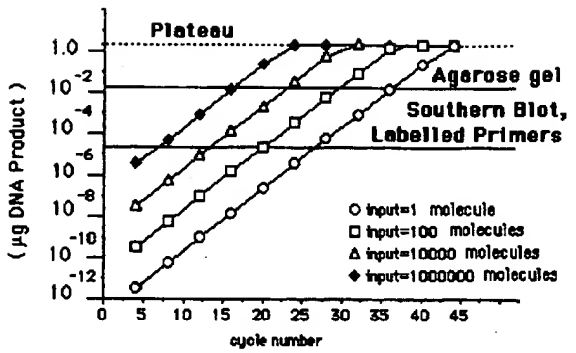

FIGURE 3. DNA product accumulation by PCR and its detection. DNA product accumulation through successive PCR cycles is shown in microgram and is estimated, assuming 100% efficiency of amplification, starting from either 1, 10^2, 10^4, or 10^6 molecules. For DNA fragment of 200 bp, one copy is equal to 2.2×10^{-10} ng. DNA product saturates in PCR at about 20 ng/µL or 2 µg per 100 µL reaction. The amplified PCR product can be detected by three methods: agarose gel electrophoresis in the presence of DNA binding dye ethidium bromide (20 ng to 2 µg), Southern blotting followed by solution hybridization with radioactive or chemoluminescent DNA probes (20 pg to 20 ng), and finally PCR amplification with radioactive or fluorescent labeled primers (1.0 pg to 20 pg). In practice, the sensitivity of using labeled primer, even though reaching a specific activity of about 50 cpm/pg of PCR product, is about the same as that of using Southern blotting, as only 1/20th of the reaction volume (or about 2.5 µL), as compared with agarose gel electrophoresis, can be analyzed by acrylamide gel electrophoresis. A contaminating source of input DNA in the aerosolized droplets may be 10^4 to 10^5 molecules. This level of contamination may be visualized after 30 cycles of amplification using ethidium bromide staining of the agarose gel.

beyond which the amplification process reaches a plateau as the efficiency of amplification progressively and rapidly decreases. Higher numbers of PCR cycles are required to reach this saturation point if fewer DNA molecules are used at the start. For DNA fragment of 200 bp, one copy is equal to 2.2×10^{-10} ng. DNA product in PCR saturates at approximately 20 ng/µL or 2 µg/100 µL reaction volume. The amplified PCR product can be detected by three methods: agarose gel electrophoresis in the presence of DNA binding dye ethidium bromide (20 ng to 2 µg), Southern blotting followed by solution hybridization with radioactive or chemoluminescent DNA probes (20 pg to 20 ng), and finally PCR amplification with radioactive or fluorescent labeled primers (1.0 pg to 20 pg). Given an input of 10^4 DNA molecules, it can be calculated that using 20 cycles of PCR amplification, assuming an efficiency of amplification of 1 (E=1), the reaction product could then be visualized by the Southern blotting, while using 30 cycles of PCR amplification allows product visualization by ethidium bromide staining of the DNA products size-fractionated by agarose gel electrophoresis.

By optimizing the conditions necessary to achieve this efficiency, it has been shown that it is feasible to use standards in separate tubes during the same RT and subsequent amplification procedure.[16] When the standards are *in vitro* synthesized RNA, quantitative PCR is possible and practical for mRNA. Using this method, multiple different messages in multiple samples can be simultaneously amplified and quantitated using a single set of standard.

Carryover Contamination in PCR: Approaches for Decontamination

Because of the extremely sensitive nature of the PCR process, contamination from carrying over of previously amplified PCR products in the same laboratory can be a serious problem. This problem affects both the diagnostic and the quantitative PCR and may be the most formidable problem in PCR application today. The contamination problem is of two types: intersample contamination during sample processing and reagent contamination from carryover. Intersample contamination is not usually the major problem but can occur if sample processing involves the use of common, nondisposable instruments, such as a tissue homogenizer or tissue-section apparatus. Reagent contamination from carryover is the major source of contamination and is detected when a simultaneously run negative-control reaction, without any added DNA template, shows a reaction product. This contamination problem should not be equated with that of a false-positive result. An intersample contamination is more difficult to identify, unless the PCR products provide multiple pieces of information, such as in alleleic typing.[17,18] The lower limit of detection using ethidium bromide staining of DNA in agarose gel is about 20 ng. To detect contaminating DNA using 30 cycles of PCR means that there must be a contaminating input DNA of 10^4 to 10^5 molecules (Fig 3), which can be present in the aerosolized droplets produced during pipetting or other common laboratory procedures. Beyond 30 cycles of PCR, even minute

amounts of contamination, such as 10 to 100 molecules, may result in visible product using agarose gel electrophoresis and ethidium bromide staining. Although in some studies, such as that with single-sperm PCR,[5,19] tandemly performed PCR with greater than 60 cycles of PCR in total has been used, it should be evident that, in most cases, more than 35 cycles of PCR may invite spurious and misleading results.

Precautionary measures such as separate work bench and micropipetters, aerosal-guarded micropipette tips, and diligent practice of aliquoting all PCR primers and reagents beforehand are absolutely necessary and integral parts of performing PCR to reduce the carryover contamination problem, which can be lessened but not prevented, in a laboratory that performs the same PCR reaction repeatedly (*eg*, a diagnostic laboratory), and it is made worse when the same laboratory performs purification of a rich source of the template material concurrently, such as a bacterial plasmid containing the DNA of interest. Fortunately several new developments appear to decrease the contamination problem.[20-22]

Sarkar and Sommer[23,24] first pointed out the possible utility of using ultraviolet light (254+300 nm) to inactivate as much as 30 ng (10^{13} copies) of contaminating double-stranded DNA. Short, contaminating DNA templates 100 to 200 bp in length are less efficiently removed[25] and the irradiation procedure has to be done before the addition of true DNA template materials, Taq polymerase, and primers. Psoralens and isopsoralens are tricyclic compounds that can be activated by 320 to 400 nm of light to cross-link with DNA molecule following DNA amplification. Cimino et al[26] and Issacs et al[27] showed that 6-aminomethyl-4,5'-dimethylisoralen (6-AMDMIP) can eliminate 10^8 copies of contaminating DNA. Clinical studies suggest psoralens can be used as effective agents for decontaminating PCR carryover of 10^9 to 10^{10} molecules.[20,22]

A second approach to eliminate contaminating DNA from carryover is to include a modified nucleotide in the PCR reaction. The substitution of deoxyuridine triphosphate for deoxythymidine triphosphate in PCR results in a DNA product that is degradable by the enzyme uracil DNA glycosylase. Longo et al[28] showed that this approach eliminated 10 ng of contaminating DNA, and Rys and Persing[22] showed the elimination of 3×10^9 copies of contaminating DNA. The use of a 3'-terminal ribose residue in DNA primers can generate a cleavable ribonucleotide linkage by RNase or by alkaline hydrolysis.[29] This system has been reported to inactivate 10^4 to 10^9 copies of DNA molecule.[22,29] This approach is useful but must be set in motion at the initiation of a PCR project.

A third approach is to use restriction enzymes to remove DNA contaminants from RT-PCR.[30] Following reverse transcription, the cDNA is treated with a restriction enzyme that cuts within the DNA segment to be amplified, but not within the primer sequences. Because the restriction enzyme is specific for double-stranded DNA, the contaminating DNA product, and not the RNA/cDNA hybrids, are eliminated. This procedure is reported to reduce contamination by a factor of at least 10^7 or more.[30] No clinical application for this method has been established, but it may be a means of diminishing carryover or genomic DNA contamination when performing quantitative PCR of mRNA.

PCR in Genetic Linkage

The locus (location on a chromosome) of hereditary diseases may be identified by linkage analysis. Disease traits that cosegregate with a DNA marker are likely to be physically linked on the same chromosome. The presence or absence of a restriction endonuclease recognition site, which can be due to a single base change at a certain site of the genomic DNA, is the basis of a DNA marker termed restriction fragment length polymorphism (RFLP), and is found to be useful to identify gene loci responsible for genetically transmitted diseases.[31] DNA extracted from lymphocytes is digested with a restriction enzyme that will specifically cut at a unique DNA sequence (usually four bases or six bases). The cut DNA is then analyzed by Southern blotting using a DNA probe from a defined genomic locus. The alleles detected by a given probe in a pedigree are revealed as different-sized DNA fragments that are complementary to the probe, inherited in a mendelian pattern. Most RFLPs are dimorphic (presence or absence of a restriction endonuclease site) and may be noninformative in a pedigree analysis if the critical individual is homozygous with this marker. A polymorphic marker will be more informative. The human genome contains many dispersed tandem-repetitive DNA sequences that are polymorphic due to a variation in the copy number of tandem repeats. Those with longer motifs (*eg*, 15 to 60 bp) are called "minisatellites"[32] while those with shorter motif (*eg*, 2 to 4 bp) are called "microsatellites".[33,34] Microsatellites are distributed approximately every 30,000 bp.[35] These loci with short tandem repeats have been found useful in constructing a detailed linkage map of the human genome.[35-39] PCR-based microsatellite analysis is extremely powerful for two reasons: first, the alleles are highly polymorphic and informative so that small pedigrees can be useful for genetic analysis (Fig 4); second, the PCR-based analysis has a turn-around time of 1 day or less, as compared with 4 to 6 days for RFLP analysis, and is much less labor intensive. An initial characterization of 813 polymorphic loci containing (C-A)n repeats was estimated to span 90% of the human genome.[37] A more detailed map complemented by different microsatellites, minisatellites, and

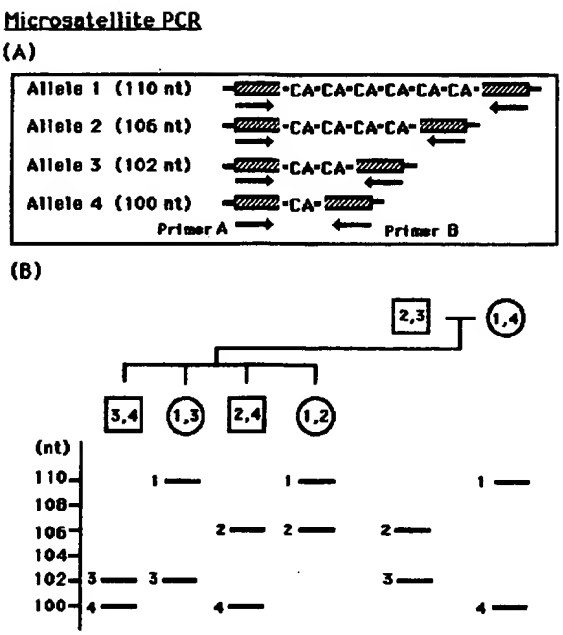

FIGURE 4. Linkage analysis using microsatellite PCR. *Top, A:* the alleles in the parents are respectively labeled as allele 1, 2, 3, and 4 containing the hypothetical $(C\text{-}A)_n$ repeats at respectively 6, 4, 2, and 1 copies of C-A repeat at this genetic locus. *Bottom, B:* the flanking PCR primers A and B define PCR products of 110, 108, 106, and 102 nucleotide (nt) in length, respectively, for each of the above alleles and are analyzed by polyacrylamide gel (sequencing gel) electrophoresis. The mendelian segregation and inheritance of the different alleles are shown.

RFLPs has recently been published, which is about 1 centimorgan apart (or roughly 10 million bp).[40,41]

Polymorphic repeat regions may show highly informative alleles. For example, a tetranucleotide repeat region around the gene for myelin basic protein demonstrated the presence of eight different alleles among 14 multiple sclerosis families and was used to exclude a linkage between this candidate gene and the disease.[42] In contrast, using the same strategy and a dinucleotide repeat flanking the angiotensinogen gene, Caulfield et al[43] found significant linkage and association of this gene to essential hypertension in 63 families. The usefulness of PCR in the elucidation and understanding of a genetic disease is best documented by the success in the study of the following diseases processes, all of which utilize the PCR technology extensively in both the linkage analysis and DNA diagnosis of the diseases: myotonic muscular dystrophy,[44] familial hypertrophic cardiomyopathy,[45-47] cystic fibrosis,[48] and most recently the inherited colorectal[49] and breast cancers.[50]

APPLICATIONS IN CLINICAL MEDICINE

PCR in Infectious Diseases

Because of its high sensitivity, PCR may facilitate the diagnosis of infectious diseases caused by viral, bacterial, fungal, protozoal, or other infectious agents. For viral-mediated diseases, the determination of the presence and quantity of a specific virus may be important in the delineation of the prevalence and natural history of a viral disease, and in following the effect of therapeutic intervention. In infectious diseases caused by bacteria or fungi, PCR diagnosis may be more expedient or cost-effective. The following examples describe the usefulness of PCR in diagnosing infectious diseases.

Using PCR on peripheral blood mononuclear cells from persons infected with HIV (as determined by conventional serologic markers), HIV-1 sequences were detected in 100% of specimens that contained discernible virus by culture and in 64% of specimens that were culture negative.[51] RT-PCR shows that viral RNA was expressed throughout the disease.[52] Clinical progression was found to be associated with an increased expression of viral genomic RNA.[52] High levels of HIV-1 RNA were found in plasma during all stages of infection, and this measure of viral burden exceeded the virus titer by an average of 60,000-fold.[13] These results suggest that a quantitative PCR approach may be useful in following the progression of AIDS. In contrast to this, the clinical usefulness of using PCR for the diagnosis of HIV-1 infection, particularly before the development of seroconversion, has not been demonstrated. The current consensus is that serologic testing defines the presence or absence of HIV-1 infection.[53]

Human cytomegalovirus infection occurs in 50 to 70% of heart transplant recipients. Peripheral polymorphonuclear leukocytes represent the main source of virus during acute infection. PCR can detect viral DNA in blood 7 to 10 days earlier than antigenemia and viremia.[54] The disappearance of viral DNA, as assessed by PCR, lags weeks to months behind the other two classic indicators. Positive results by PCR alone were not associated with overt clinical symptoms.[54] These results suggest that PCR positivity without quantitation of virus load may not add to the clinical management of posttransplant cytomegalovirus infections.

Viruses have long been suspected to be causal factors in some cases of myopericarditis and dilated cardiomyopathy.[55-57] In a population of patients with dilated cardiomyopathy with suspected prior myocarditis, 10% (5/48 patients[58]) and 20% (1/5 patients[59]) of samples were found to be positive for enteroviral RNA by PCR. These findings may be important in that in a murine model of dilated cardiomyopathy induced by encephalomyocarditis virus, positive viral cultures were not detected from hearts 14 days postinfection, but viral RNA was detected by PCR in about 30% of the hearts at 90 days.[60] However, at present, the routine clinical use of the PCR assay for the detection of virus

in idiopathic dilated cardiomyopathies is not practiced or advocated.

The laboratory diagnosis of *Mycobacterium tuberculosis* by PCR may facilitate the clinical management of tuberculosis. PCR amplification has been applied to different regions of the mycobacterial genome such as the 65-kDa protein, MPB64 protein, antigen β, repetitive sequences and r(ribosomal)RNA,[61-67] and it was found to be applicable to a variety of specimens such as sputum, urine, feces, gastric aspirate, CSF, blood, bone marrow, lymph node biopsies, and abscess aspirates. The amplified DNA fragment can be further hybridized with species-specific oligonucleotide probe for the identification *M tuberculosis* complex, *Mycobacterium avium-intracellulare* complex, or *Mycobacterium fortuitum*.[64,68,69] Large-scale application in the clinical setting, however, shows that the sensitivity and specificity of this diagnostic approach remain uncertain. Therefore, as pointed out by a US Public Health Service report, PCR approach cannot at present replace the established techniques for the diagnosis for *M tuberculosis*.[70]

PCR detection of microorganisms appears to be broadly applicable. Examples of organisms that have been identified using this technology include *Pneumocystis carini*,[71] *Candida albicans*,[72] *Mycoplasma pneumoniae*,[73] *Bordetlla pertussis*,[74] and *Aspergillus fumigatus*.[75,76] The routine use of PCR-based diagnosis of these and other organisms will have the same uncertainty of diagnostic accuracy as that faced with *M tuberculosis*. In addition, PCR diagnosis of infectious agents would require an *a priori* supposition of the type of organism, in contrast to microbiologic culturing techniques that are all inclusive. Multiplex PCR (multiple primer sets in the same PCR reaction) may circumvent some of these problems, especially if only a limited number of organisms are to be assayed and excluded from a compartment that is normally sterile, like the CSF. Finally, if the organism is an opportunistic pathogen that colonizes the normal tissue, such as *P carinii* in the lung,[53] PCR assays may not add to the conventional tests or may result in false-positive diagnosis.

PCR in Epidemiology

The ability to determine or "type" a single strain of bacterium or virus is the basis of epidemiologic attempts to identify the source of an infectious disease outbreak. The classic approach, which continues to be the cornerstone for differentiating strains, is based on phenotype analysis, such as serotyping, biotyping, antibiotic susceptibility typing, and bacteriophage typing. These approaches aim to identify shared characteristics of isolates that are derived from the clonal expansion of a single precursor organism. These phenotypic methods are limited by the reproducibility and, especially, the discriminating power of the methods.[77] DNA-based approaches include plasmid profile analysis, restriction endonuclease digestion followed by Southern analysis of chromosomal DNA (using either conventional gel electrophoresis technique for size-fractionation or using a "pulse field" gel electrophoresis technique that uses alterating changes in polarity of electrofield to separate large pieces of DNA fragments), and PCR-based DNA analysis.[77-79] Another promising new approach of PCR typing involves the use of repetitive elements in the bacterial genome. PCR amplification performed using DNA primers matching such repeated DNA elements, or "Rep-PCR," has been reported to produce strain-specific fingerprints useful for clonal identification.[80-82] These genotyping methods are technically less demanding and easier to maintain (for example, bacteriophage typing requires the maintenance of bacterial and viral cultures for comparison and for identification) than the phenotyping methods. Theoretically, PCR-based DNA analysis, perhaps in conjunction with some type of DNA sequence analysis, promises to be most sensitive and most discriminatory, if the organism understudy has already been identified. The potential limitation of PCR-based epidemiology approach is again due to its extreme sensitivity and possibility of carryover contaminations.

PCR in Neoplastic Diseases

The histopathologic diagnosis of cancer is influenced by the expertise of the pathologist, but it usually is established when at least 1 to 10% of the cells in the tissue sample are neoplastic.[83] Molecular detection of DNA rearrangements, point mutations, deletions, and DNA amplification may be more objective and with greater sensitivity for establishing a diagnosis. In addition, molecular diagnosis may be used for staging the disease, assessing prognosis, evaluating the effect of a therapeutic intervention, and identifying populations at risk.

In chronic myelocytic leukemia, a translocation event occurs in 95% of cases, producing the hallmark of a Philadelphia chromosome.[84,85] The translocation joins the 5′ portion of the breakpoint cluster region gene located on chromosome 22 with the 3′ portion of the c-*abl* gene on chromosome 9.[86-88] PCR has been used to detect the presence of the fusion breakpoint cluster region-*abl* mRNA following RT, even in Philadelphia chromosome-negative chronic myelocytic leukemia.[89,90] In lymphoma, clonal lymphocyte proliferation occurs with uniform antigen receptor gene rearrangement that can be assessed by Southern blot hybridization or PCR. PCR is performed using oligonucleotide primers complementary to conserved sequences in the V and J segments of immunoglobulins or T-cell receptor genes. The joining of V-(D)-J DNA

sequences occurs through normal recombination during the differentiation of B and T lymphocytes. Such a PCR amplification performed on polyclonal lymphocytic population results in smear in agarose gel electrophoresis whereas discrete bands occur when the lymphocytes contain clonal population present in lymphomas. PCR has been used to detect residual tumor,[91,92] although its utility in management of the disease is not yet established. In follicular lymphoma, over 90% of cases have the t(14;18) translocation, joining the immunoglobulin heavy chain gene on chromosome 14 to the gene for BCL2, which regulates apoptosis (programmed cell death). PCR has been used to detect residual disease[93,94] and to follow the success of cancer cell purging in autologous bone marrow transplantation for follicular lymphomas.[95]

The molecular biology of lung cancers shows the interaction of multiple genetic changes involving oncogenes.[96-98] Small cell lung carcinoma (SCLC) is found to be associated with amplification of the *myc* family of proto-oncogenes. The *ras* family of proto-oncogenes is mutated in 20% of non-small cell lung carcinoma (NSCLC). Mutations of the p53 gene have been found in association with both SCLC and NSCLC. In addition, microsatellite instability, detectable by microsatellite PCR, has recently been found to be present in 9% of NSCLC and 50% of SCLC.[99] It has been demonstrated that screening with conventional techniques for lung cancer in high-risk populations using routine chest radiograph and sputum cytologic study does not alter the disease survival (reviewed by Szabo et al[97]). Whether earlier detection by using PCR-based molecular diagnosis will change the prognosis is not yet known. At present, no systematic approach using PCR or other molecular technology in the presymptomatic diagnosis of lung cancer has been established.

PCR in Genetic Diseases

Genetic diseases such as Tay-Sachs and β-thalassemia have been targeted for screening and counseling since the 1970s resulting in a drastic reduction in the incidence of these diseases.[100] In recent years, genes associated with many familial diseases have been identified, including those for cystic fibrosis, fragile X syndrome, myotonic dystrophy, spinal muscular atrophy, hypertrophic cardiomyopathy, and Duchenne's muscular dystrophy (DMD). In addition, some cancer-causing genes have been deduced and cloned, such as the Rb gene associated with retinoblastoma, APC gene associated with adenoma polyposis coli, mismatch repair genes for nonpolyposis colonic cancer, and BRCA1 for breast cancer. Because of its high sensitivity, PCR-based screening has been envisioned to be particularly useful for these genetic diseases. The successful application of PCR in genetic diseases diagnosis appears to be dependent on the degree of complexity of molecular abnormalities associated with a disease. Within a clinically and phenotypically defined disease process (such as the hypertrophic cardiomyopathy, DMD, cystic fibrosis, and hereditary breast and ovarian cancer), heterogeneous molecular defects are found that may be mutations at different areas of the same gene or that may be mutations of entirely different genes.

DMD affects 1:5,000 male patients and is primarily due to a deletion of a gene fragment at DMD locus on the X chromosome (Xp21). Chamberlain et al[101] have developed multiplex PCR in which multiple sets of primers are used simultaneously in the PCR reaction to detect 80 to 90% of all DMD gene deletions. This procedure greatly facilitates the diagnosis of DMD disease and in many cases replaces muscle biopsy in the diagnosis of DMD.[102]

In cystic fibrosis, the gene cystic fibrosis transmembrane conductance regulator gene is the site of disease-causing mutations. The predominant mutation, originally identified in 1989, is ΔF508, which accounts for 70% of the mutations.[103-105] However, by 1992, more than 170 mutations had been identified, and by 1993, over 350 mutations had been reported. The large number of mutations that can result in cystic fibrosis makes a screening program a difficult task.

Familial hypertrophic cardiomyopathy is an autosomal dominant disorder with myocardial hypertrophy and myofibrillar disarray. Scores of different mutations in different exons of β-cardiac myosin heavy chain, which is located on chromosome 14q1, have been demonstrated to be present in different families.[106-109] In addition, multiple other genetic loci, including, 11p13-q13,[110] 1q3,[111] and 15q2[112] have been identified to be linked with the disease. The disease locus on 15q2 and 1 q has now been identified, respectively, to be the α-tropomyosin and the cardiac troponin T gene.[47] Thus, this genetic disease syndrome, with a similar clinical manifestation that consists of myocardial hypertrophy, myofibrillar disarray, and sudden cardiac death, is composed of families with diverse molecular mutations in different genes. Consequently, a PCR-based genetic diagnosis will not be simple and may not be all inclusive.

Thus, from studying the various forms of genetic diseases, it may be surmised that the diagnosis of a genetic disease by PCR will not be always straightforward; a PCR-based diagnosis test will be useful only in that a positive test result is highly discriminatory but a negative test result is only relevant to the genetic mutation that has been tested. In other words, within a characterized pedigree and thus a known genetic mutation, a PCR-based diagnosis will be useful; the same test, however, will have limited usefulness for population screening. With some exception, PCR-based

method will be limited for broad clinical screening in that a large number of primers are needed for each disease entity and that an additional cloning or direct DNA sequencing reaction may also be needed.

Future Applications of PCR

PCR application in basic and clinical medicine in the literature has appeared to increase without bounds, attesting to the power of this technique. Although the power of this technique resides in its ability to detect a single molecule of DNA, this extreme sensitivity underlies its main limitation. For diagnostic purposes such as the detection of a disease-causing agent, be it an infectious organism or a mutated gene, the containment of PCR carryover becomes an area of primary importance. Thus, "routine" PCR-based diagnostic tests performed by individual laboratories will not be uniformly accepted at this time.

A second problem of PCR-based diagnostic tests is also related to its sensitivity. Detection of an abnormal gene or transcript might not be clinically relevant, as this abnormality may not be pathogenetic. A quantitative analysis may at times be able to overcome this problem. The technique requires proper standards and controls for valid interpretation. It is anticipated that well-executed quantitative data will become useful information that may ultimately help us to understand the physiologic and the pathophysiologic processes at the molecular level.

The utility of PCR in the screening of specific genetic disease will increase as more disease loci and genetic elements are identified. In this area, the ethical problem is a separate area of consideration. The use of PCR in the diagnosis of hematopoietic malignancies at present complements but does not replace the standard diagnostic tests. The screening of multifactor malignant disease, such as carcinoma of the lung, in a susceptible population, is theoretically possible but presently neither realized nor tested for its clinical applicability. The recent realization that many different types of cancers show microsatellite alterations suggests a new approach for the detection of human cancer. The screening of disease susceptible genes, of which breast cancer and colonic cancer may be candidate diseases, will likely be part of the arsenal for preventive medicine. The utility of PCR in epidemiology is an important new addition to this field although it will be limited in some settings by its requirement that the infectious agent will have to be first identified and region of unique sequences established. In this respect, Rep-PCR may be particularly useful. With the continuous innovations seen with this technology, PCR is now and will continue to be a powerful and an indispensable technology in basic research and in diagnostic medicine.

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Control of Contamination Associated with PCR and Other Amplification Reactions

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INTRODUCTION TO CONTAMINATION CONTROL

A molecular diagnostic laboratory that plans on using one or more in-vitro amplification reaction(s) (IVAR) should also be evaluating measures to control a contamination problem which parallels the use of these procedures. Historically, the concept of contamination in a biomedical laboratory has been associated with the unintentional disbursement of radioisotopes (e.g., 32P, 35S, etc.) in areas not designated for their use. Removal of these radioactive species was typically straightforward, rapid, and effective. Indeed, trace amounts of these radioactive species have usually been removed without problem and the laboratory restored to its original non-contaminated condition.

However, the molecular genetics laboratory that has become contaminated with a biological species faces a much more difficult problem. Since the advent of the polymerase chain reaction (PCR)*, the molecular genetics laboratory has possessed an experimental capability of enormous sensitivity. Unfortunately, the PCR's exquisite capacity for amplification was accompanied by its extreme sensitivity to the presence of its own product as a feedback contaminant. [1-3] Because of the need for some molecular diagnostic laboratories to routinely detect less than 100 copies of certain target templates (e.g., viruses), this susceptibility of the PCR to trace amounts of its own product means the laboratory has a continual requirement to control a species it cannot easily detect, see or readily remove.

Although the two types of contamination (e.g., radioactivity and amplified nucleic acid) can both be considered as being unintentional, any further comparison disappears almost immediately on at least three accounts:

A. **Longevity:** The most common radioisotope used in molecular biology has a short half-life (e.g., TH for 32P = 14.3 days). As such, by waiting a certain period of time following the contamination event (6 x TH), the major amount (> 98%) of the radioactive contaminant spontaneously degrades. By contrast, the main product (and corresponding contaminant) produced by the PCR (e.g., dsDNA) has been shown to exist for thousands of years.4 However, since some of the IVAR produce RNA instead of DNA, their products (as contaminants) will exist for much less time due to the inherent instability of RNA. Thus, IVAR that produces RNA may not present as much of a serious problem. Unfortunately, contamination produced by an IVAR that yields dsDNA can be considered permanent. Laboratories have physically moved their facilities or stopped performing a PCR assay for particular targets (e.g., viral) because the labs were permanently contaminated and negative controls (water blanks) included in the assays were consistently positive.

B. **Detection:** Radioactive nucleotides typically used in molecular biology (e.g., 32P, 35S, etc.) emit beta-particles that induce a response from a Geiger counter. By contrast, nucleic acid molecules, which are produced by an IVAR and then accidentally disbursed, are virtually impossible to detect by direct means. (Note: The exception to this is when the PCR [or

other IVAR] utilizes a radioactive precursor such as one of the dNTPs or a radiolabeled amplimer to produce a radioactive amplicon.) **Amplification:** Radioisotopes do not have the intrinsic property of reproduction. Rather, the amount of radioactivity in a labeled species always begins at a fixed amount and is decreased until the last of the species has undergone the corresponding decay reaction. IVAR products, by contrast, can be reproduced to orders of magnitude (104 to 109) beyond their original concentrations. Moreover, the amount of DNA product present in even minuscule amounts (e.g., nanoliter, or less) may be sufficient to serve as a template for subsequent IVAR (e.g., PCR, LCR, RCR, etc.). (Table 1)

Table 1

In Vitro Amplification Reactions

Name	Abbreviation	Amplified Product	Enzyme(s) Needed	Reactants Needed
1. Polymerase Chain Reaction	PCR	DNA	tsDNA polymerase	4 dNTPs + 2 oligonucleotides
2. Ligase Chain Reaction	LCP	DNA	tsDNA ligase	ATP + 4 oligonucleotides
3. Repair Chain Reaction	RCR	DNA	tsDNA polymerase tsDNA ligase	(dATP and dCTP) or (DTTP and dGTP) + 4 oligonucleotides
4. Self-Sustained Sequence Replication	3SR	RNA	Reverse transcriptase RNA polymerase	4 dNTPs + 4RTPs + 2 oligonucleotides

Note: ts = thermally stable
dNTPs = dATP, dCTP, dGTP, dTTP
RTPs = ATP, CTP, GTP, UTPFor example, consider a first round PCR (volume = 100 5l) that started with 100,000 copies of a single copy gene contained in 1 5g of genomic DNA. When this template DNA is subsequently amplified by a factor of 105 (nominal for the PCR), every nanoliter would contain 100,000 copies of amplicon. If a single 20 micron diameter aerosol droplet (volume = four picoliters) from this first round PCR tube is inadvertently introduced into the tube for the next PCR, there are sufficient numbers of DNA amplicons (e.g., ~400) to

serve as templates for the next PCR and thereby yield a detectable reaction product in that tube. Obviously, controlling aerosols containing droplets of less than 50 microns in size is critical for preventing this type of contamination. This is especially true if a high number of thermal cycles is used for the PCR.

METHODS TO CONTROL CONTAMINATION

At present, there are a number of generally recognized procedures that have been reported with regard to controlling IVAR contamination.[5-9] These procedures will be discussed later in greater detail. The following section will focus on more recent methods that have been specifically developed to minimize or eliminate contamination due to amplicon carryover. These newer methods can be grouped in two broad categories: pre- and post-amplification treatments of the reaction tube contents. It should be mentioned, however, that no effective method is known which can remove an IVAR amplicon from a contaminated laboratory. In this case, the choices are limited to abandoning either the assay for that particular template or the laboratory where the template is currently assayed. This very unattractive choice provides strong incentives for a laboratory to deal with this problem on a prospective basis.

DETECTION OF CONTAMINATION

Before discussing contamination control procedures, a brief mention should be made about how IVAR amplicon contamination can be detected. In this context, contamination is defined as the unwanted presence of DNA (or RNA) amplicons that may serve as a future IVAR template. At present, no experimental means exist which can directly detect the ultra-trace amounts of this template (e.g., picogram amounts or less). The best way to monitor for IVAR template contamination BEFORE it becomes a serious problem is to use reagent-only ("template-negative") blanks which are prepared and included with the other samples in each IVAR run. [1,5,6] These controls contain all of the required reaction constituents EXCEPT the template (sterile distilled water can be substituted for the template). The presence of contamination is then signalled by the appearance of the test amplicon in the reagent-only blank tubes.

1. **Reagent-Only Controls:** These should also be used to check for contamination in newly prepared batches of IVAR reagents (e.g., 10X buffer, dNTPs, amplimers, etc.) before they are aliquotted or used for routine analysis. To monitor for this contamination, the new reagent is substituted for H2O in the reagent-only blank. Once analyzed in this manner and found to be free of contamination (as evidenced by the absence of IVAR amplicons), the new reagent should be dispensed into small volume aliquots and frozen. Sufficient aliquots should be thawed for preparation of one mastermix, and then discarded. Under no circumstances should individual reagent aliquots be re-used.

CONCEPTS OF CONTAMINATION CONTROL

As indicated earlier, there are two major approaches to controlling IVAR contamination. Generally, these philosophies can be applied to any of the in vitro amplification techniques. These control techniques focus primarily on activities performed before or after the IVAR has occurred. Several may actually be found under both headings as illustrated below. Considering the intense amount of interest in IVAR contamination and the profound implications of its impact on diagnostic and research applications, alternative philosophies can be expected to emerge in the future as well.

A. Pre-Amplification Contamination Control

This approach concentrates on activities that precede the actual amplification reaction. Several different methods can be found under this heading. One involves controlling or eliminating aerosols in handling and delivering liquids used in preparing reagents for the reaction.[5,6,8] Two experimental approaches have evolved for accomplishing this: 1) the use of positive displacement tips for pipettors, and 2) the use of aerosol resistant tips. Since there are numerous pipetting steps involved in setting up an IVAR, elimination of aerosols that may contain 100 to 1000 copies of the amplifiable template is paramount. A detailed examination of each device is given below.

1. Positive Displacement Pipettors: When first introduced, only one brand of pipettor used positive displacement tips (i.e., disposable plunger and barrel combination). Although these tip sets came in several sizes (e.g., 20 5l, 200 5l, etc.), they had to be manually assembled into functional units then autoclaved before use. With the advent of PCR, the demand for these pipet tips grew very rapidly and the imported tips were, unfortunately, rationed for a brief period of time. Since then, at least two other domestic pipet manufacturers have started to produce positive displacement tips which are also of two-piece construction (plunger and barrel). One of these newer brands comes preassembled and can be directly autoclaved. The use of these tips prevents the carryover which results in contamination (Figure 1). The cost for the assemble units are $0.35-0.37 each.

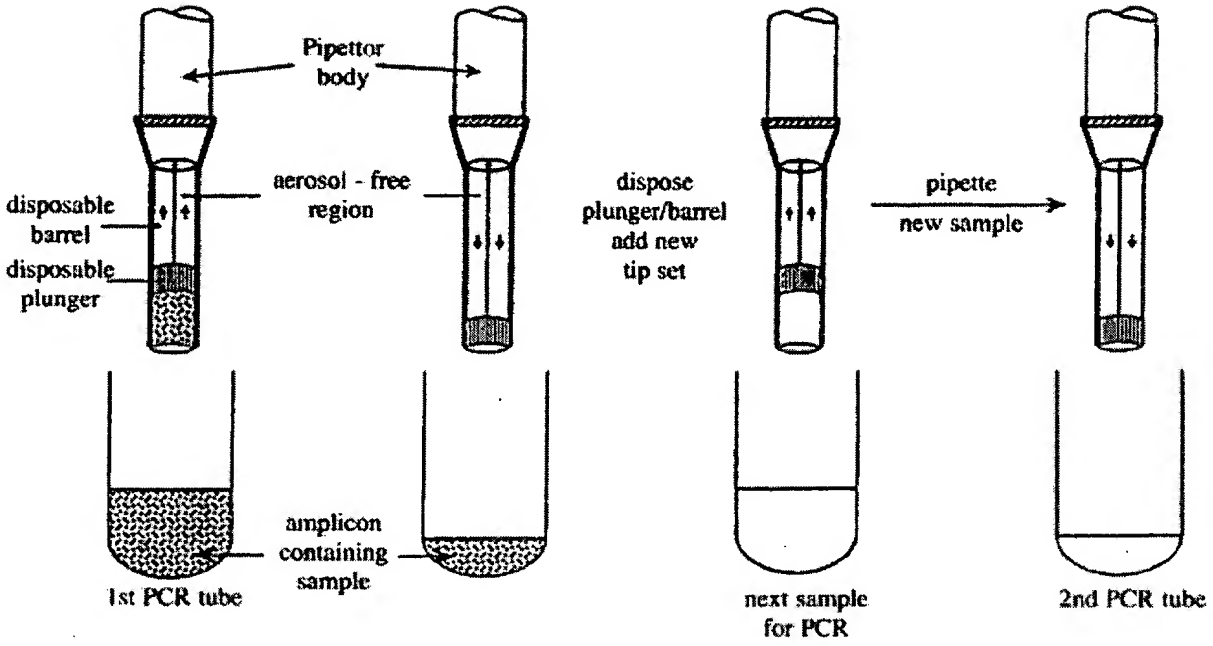
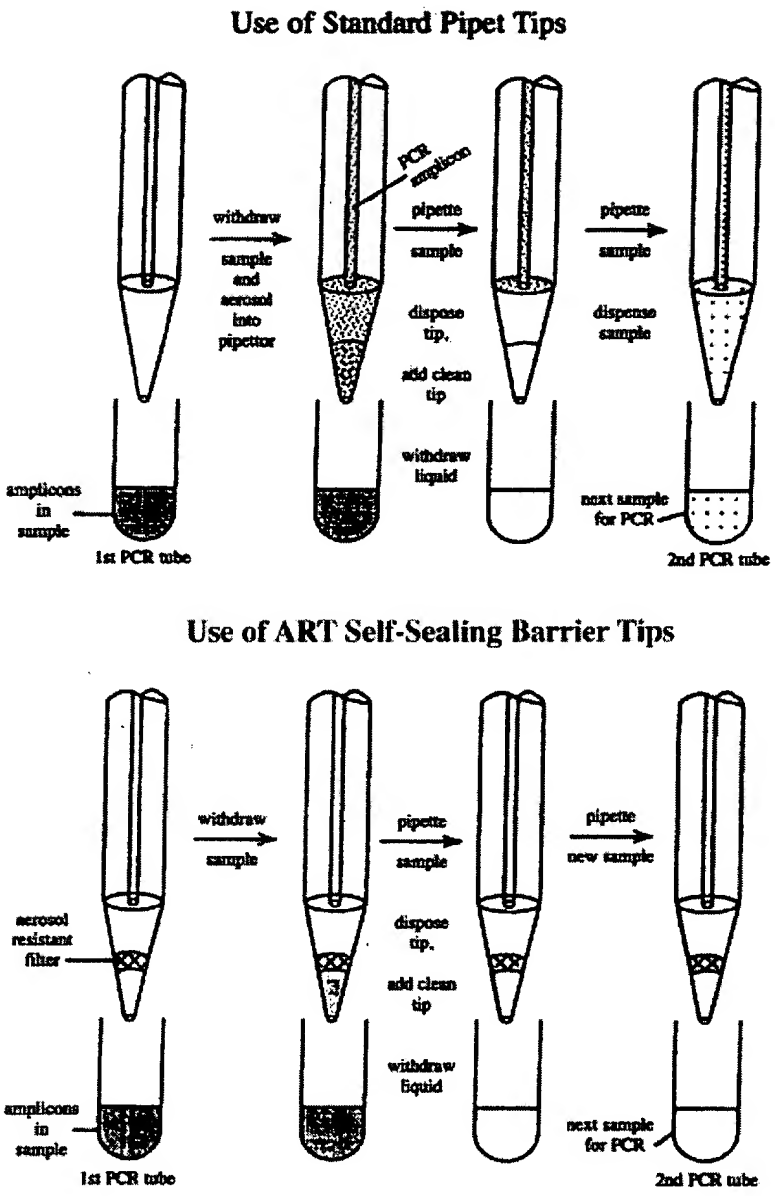


Figure 1: Use of a positive displacement tip illustrates that no aerosols are carried into the tube for the 2nd round of PCR.

2. ART Self-Sealing-Barrier Tips: As an alternative to the positive displacement tips, Molecular Bio-Products, inc. (San Diego, California), developed the Aerosol Resistant Tip (ART). These specialized tips are now available in over 20 sizes, ranging from 10 5l to 5000 5l. ART tips fit most of the commercially available air-piston pipettors and some robotic pipetting stations. The principal feature of the ART pipet tip is a porous, self-sealing, physical barrier which is located between the pipet tip's upper section and the tip's orifice. When the plunger on the pipettor is released, air flows up through the porous barrier, leaving any aerosols trapped inside the barrier (Figure 2). It is the amplicons in the aerosol which, if deposited inside the pipettor's mandrel, could contaminate the next sample during subsequent pipetting steps. To further reduce pipettor contamination, one manufacturer has introduced a pipettor which can itself be autoclaved.
   Several differences exist between positive displacement, standard and ART tips. Positive displacement tips require a special pipettor body. Although autoclavable and effective, they are expensive. Standard tips, albeit inexpensive, are not appropriate for IVAR due to problems with contamination. ART tips cannot be autoclaved due to the patented barrier, and are, therefore, pre-sterilized by electron beam radiation.



3.

Figure 2: Use of ART Self-Sealing Barrier Tips illustrates that aerosols generated during initial pipetting steps are restricted to lower portion of the tip.

Table 2

Comparison of Pipet Tips

Type of Pipet Tip	Use standard micro-pipettor	Can be autoclaved	Prevents contamination
1. ART Self Sealing Barrier Tips	Yes	No	Yes
2. Positive Displacement	No	Yes	Yes
3. Standard	Yes	Yes	No

ART tips are lot certified RNase-, DNase- and Pyrogen-free, proven to prevent contamination, and cost approximately G that of positive displacement tips (Table 2). +Note: My laboratory has used ART brand tips from Molecular Bio-Products, inc., for over a year to manipulate samples and set up amplification reactions. We find them to work extremely well.

3. Uracil-N-Glycosylase (UNG): Another pre-IVAR approach used to control contamination is an enzymatically-based method to treat samples, which are to be amplified by an IVAR. The method exploits the susceptibility of the deoxynucleotide uracil to the enzyme, uracil-N-glycosylase (UNG).[10] The contamination protocol is initiated by replacing dTTP, normally contained in the PCR mixture, with dUTP (Figure 3). This replacement has a minimal effect on the PCR since uracil can form complimentary hydrogen bonding to adenine almost as well as thymine. The dUTP is therefore readily incorporated into the amplicon produced by the subsequent PCR.
   Following completion of the first PCR, the reaction mixture with the dUTP containing amplicons can be analyzed normally. The next set of samples to be amplified using the PCR is now briefly treated with the enzyme UNG. The samples are then heated to destroy the UNG activity, and the heat-treated samples added to the PCR master mixture for the next round of PCR. During the incubation with UNG, any dUTP-amplicon that was unintentionally carried into the samples destined for the next round of amplification is subjected to enzymatic hydrolysis by UNG. The enzyme recognizes the uracil bases in both strands and cleaves the N-glycosylase bond that holds the uracil onto the deoxyribose-phosphate backbone. Cleavage (and subsequent loss) of the uracils then destabilizes the duplex strand(Figure 3).

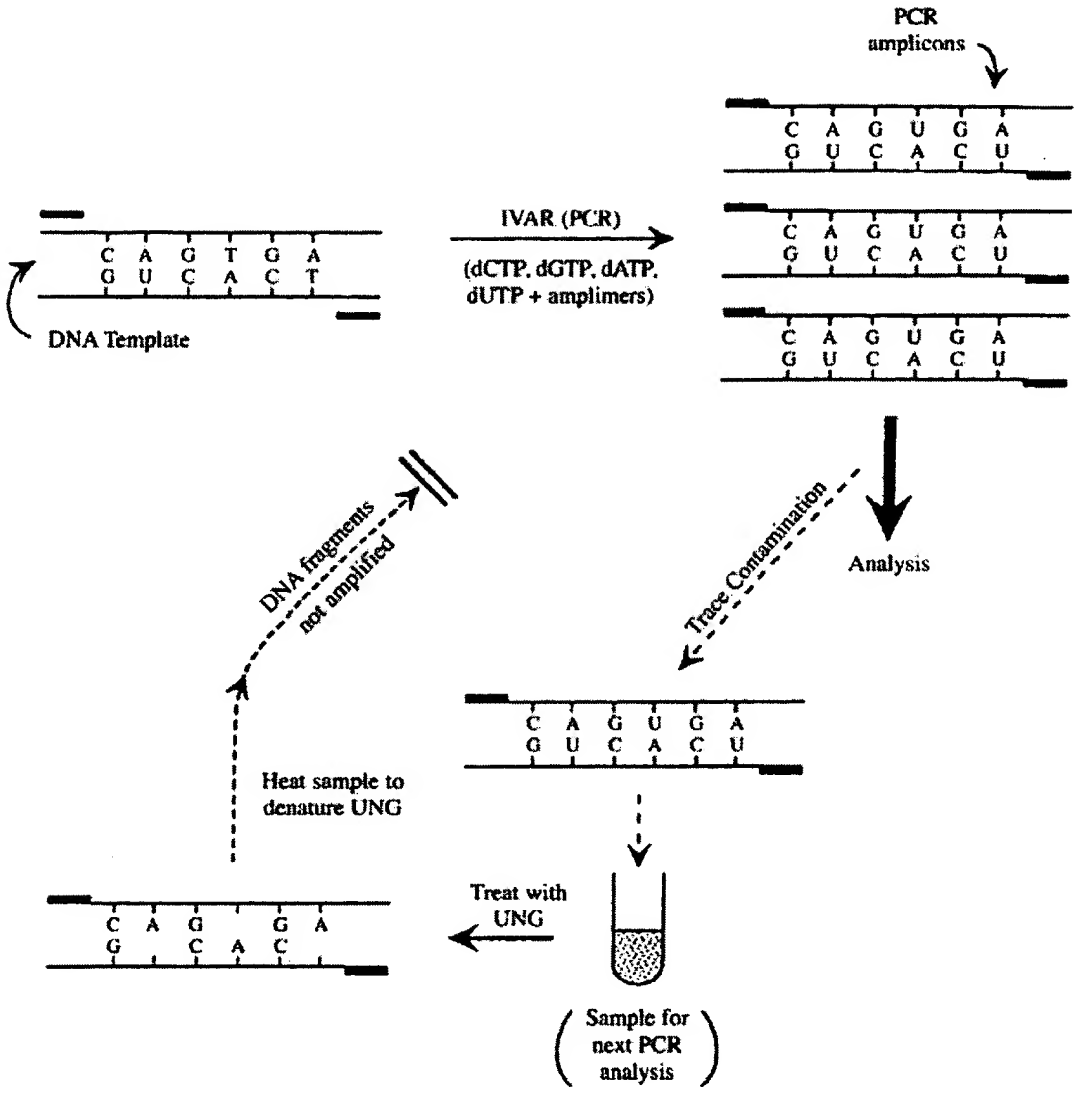


Figure 3. IVAR contamination control procedure which utilizes the enzyme uracil-N-glycosylase, UNG. The PCR amplimers bind at the sites indicated.

Several caveats are worth mentioning. First, the UNG-treated DNA is unstable to the subsequent denaturing temperatures of the next round of PCR and is degraded into smaller fragments, which are unsuitable as templates for amplification. While the replacement of dTTP with dUTP is simple, some adjustments to the PCR parameters are required and the yield of the PCR may also diminish.8 Second, the possibility that UNG may survive the heat denaturing step and degrade the product dUTP containing amplicon can occur.[11] Thornton et al found that if PCR

products were stored at either 4: C or 25: C following the end of the PCR, there was degradation (and loss) of amplicon product. Apparently, the UNG renatures and then degrades the dUTP-containing amplicon. This degradation could be prevented by adding a bacterial UNG inhibitor (Ugi) or by setting the soak cycle to 72: C after the PCR. Finally, this contamination control procedure may not work with the LCR unless one or more of the amplimers contains uracil. However, it may be useful for the Repair Chain Reaction (RCR) or the Self-Sustained Sequence Replication (3SR) because both incorporate dTTP during the course of the amplification sequence. The present cost of the UNG procedure is $1.50 per PCR tube.

4. 3' Ribonucleotide Amplimer: A newer alternative to the UNG procedure is the Triple CTM (Cross Contamination Control) technique shown below. In this pre-reaction protocol, the amplimers are synthesized with a RIBONUCLEOTIDE instead of the corresponding deoxyribonucleotide at the 3' position. The 3' modified amplimers replace the normal amplimers in the reaction mixture and the PCR is allowed to proceed. In a protocol similar to the UNG method, the samples for the next (second) round of PCR are then treated with either: 1) ribonuclease H or 2) strong base (OH-). If carryover amplicons are present, they are cleaved at the 3' end of the modified-amplimer's ribonucleotide (see Figure 4). The strand is nicked and is degraded after the first round of heat denaturation. The carryover amplicons, if present, are therefore not amplified. The manufacturer claims that this method provides a minimum of 4 orders of contamination protection. This contamination control procedure would appear to be useful for IVARs that use oligonucleotides as amplimers, which makes it suitable for PCR, LCR, RCR, and 3SR. The procedure can also function as a post-PCR control procedure. Whether this product can be effectively used for amplifying RNA-based templates, however, remains to be demonstrated. The cost for the ribonucleotide-modified amplimer technique is approximately $0.23 to $0.32 per PCR tube depending on the amount of amplimers used in each tube.

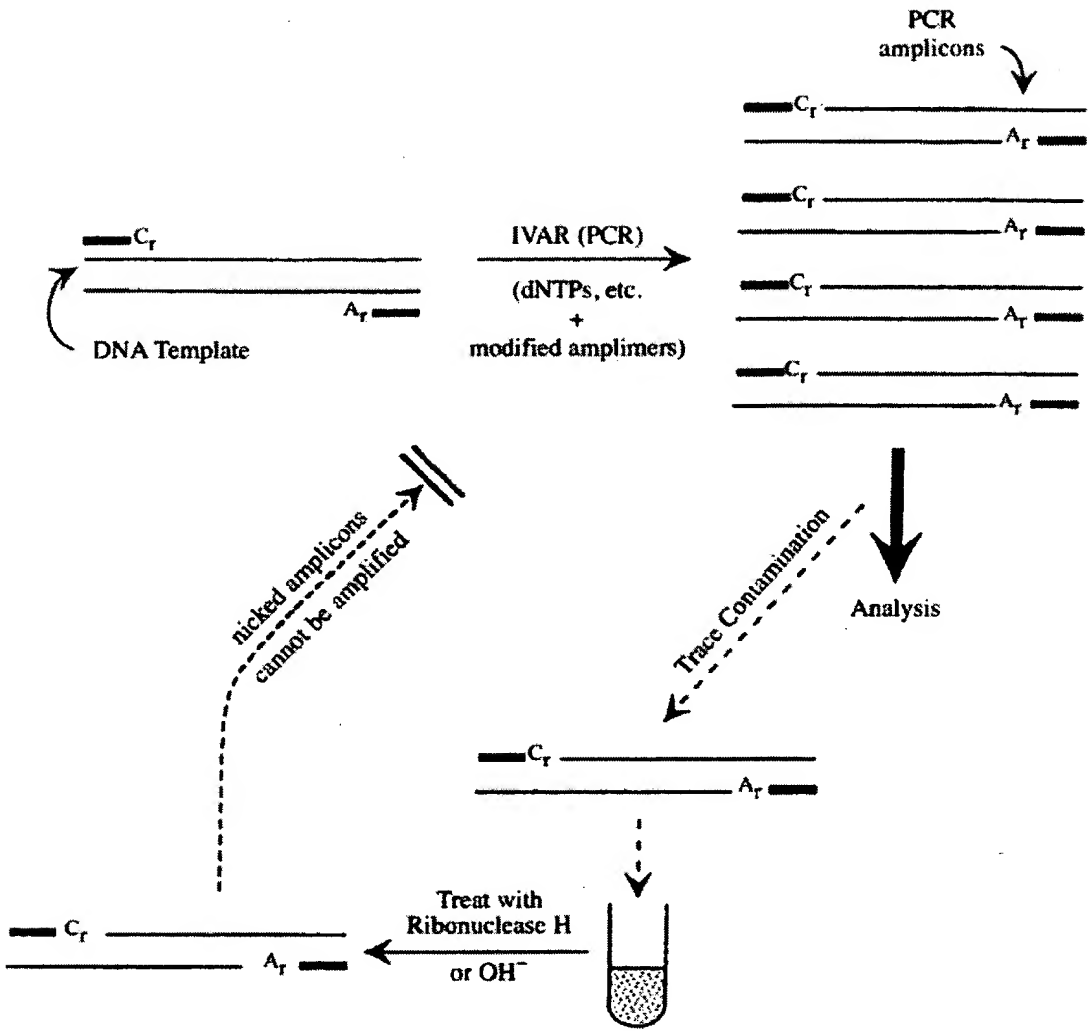


Figure 4. 3' ribonucleotide-modified oligonucleotides used for contamination control. The example amplimers contain the ribonucleotide adenien (Ar) or the ribonucleotide cytosine (Cr) at their respective 3' ends. When later attacked by ribonuclease H or OH, the phosphate:ribose bond is cleaved.

B. POST AMPLIFICATION CONTAMINATION CONTROL

One principle method is used for control of undesirable distribution of amplicons after the IVAR is completed. It is based upon the demonstrated sensitivity of DNA to ultraviolet light.12 There are two different sets of photoreactions that have been developed for POST-IVAR contamination control.

1. **UV-Induced Thymine Dimers:** In this procedure, ultraviolet light (254 to 300 nm) photocrosslinks pairs of adjacent pyrimidine bases into cyclobutane-like dimers (Figure 5). The dimers are composed mostly of

thymine::thymine (TT) dimers, although a few thymine::cytosine (TC) and a rare cytosine::cytosine (CC) combinations are formed as well. Once created, these modified structures cannot be removed from the dsDNA (or ssDNA) templates because most of the thermally-stable DNA polymerases possess little or no exonuclease activity. This contamination control procedure exploits the presence of the thymine dimers (TT) since they sterically block extension of the incomplete strand when encountered by the DNA Polymerase.

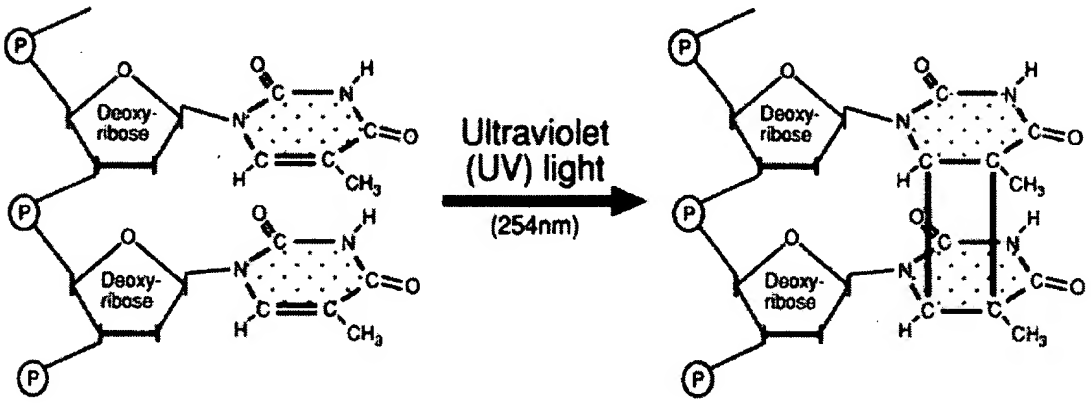


Figure 5: The formation of a thymine cyclobutane dimer in response to UV light. Similar dimers will also form between any adjacent pyrimidine pairs (e.g., CT or CC).

The extent to which the thymine dimers are formed is a statistical process dependent upon several factors, including duration of illumination and prevalence of thymines in target dsDNA. Due to some restrictions on attributes of the templates (e.g., length in bp, A:T content, frequency of adjacent thymines in DNA sequence, etc.), this technique appears to be of limited value. For example, this procedure works better for longer amplicons (e.g., ~500 bp) since the number of statistical sites for dimer formation is much greater than for short amplimers (e.g., ~100 bp).

The treatment protocol is simple. First, samples are processed by an IVAR (PCR) under normal conditions. Second, immediately after the thermal cycling reaction is completed, the unopened PCR tubes are irradiated with UV for a brief time period. Third, the samples are processed as usual [13] (Figure 6). If a UV transilluminator which emits 254 nm is used to irradiate the IVAR (PCR) tubes, the time required to effectively remove contamination has to be empirically determined, although <20 minutes may be adequate. [14,15] For DNA amplicons present in the tubes, intrastrand thymine dimers are then formed between adjacent thymines. If now accidentally introduced into a subsequent IVAR (PCR) reaction mixture, these thymine dimers inhibit extension of the single strand DNA template by sterically blocking the DNA polymerase, preventing amplification of the photoderivatized template. A potential problem of incomplete UV irradiation due to the dNTPs absorbing much of

the UV light (and hence not allowing UV crosslinking of the thymines) may be overcome by omitting the dNTPs out of the PCR master mix until after the master mix has been irradiated. [16]

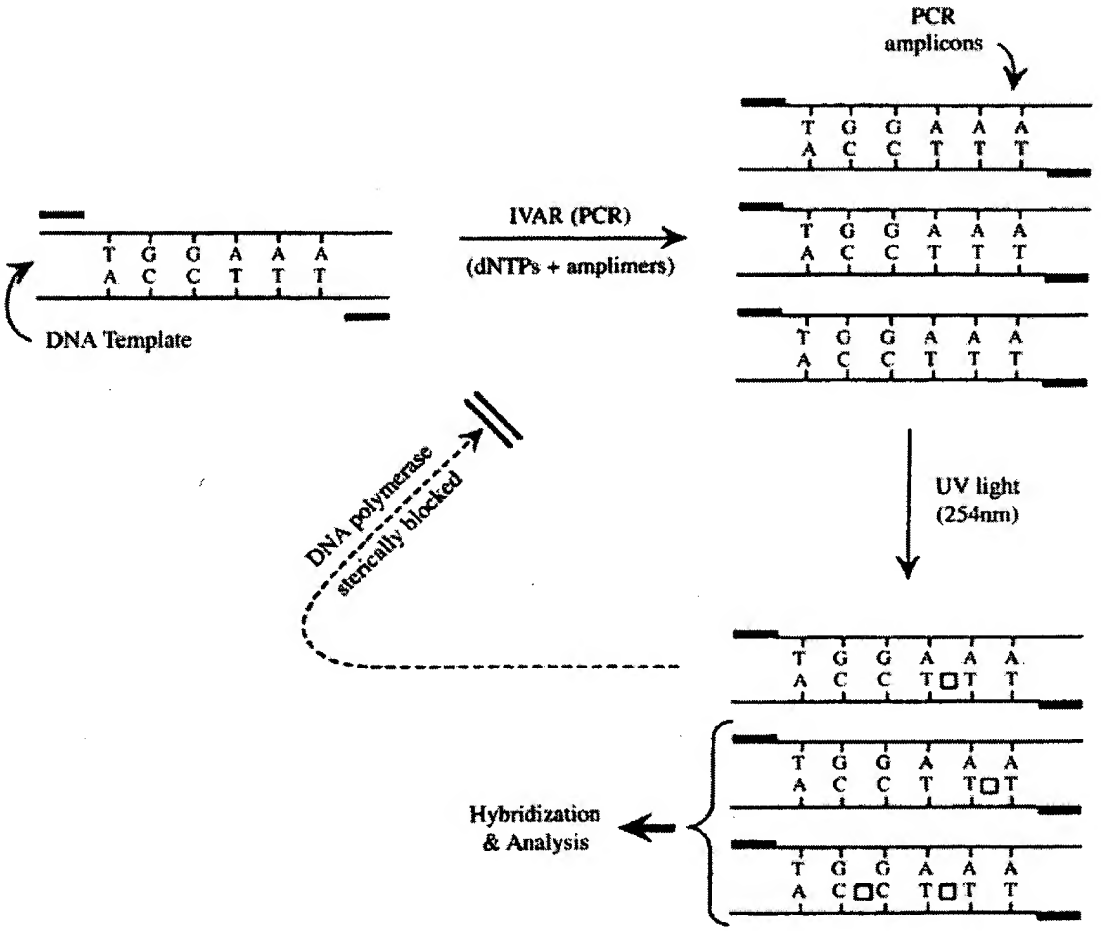
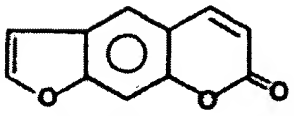


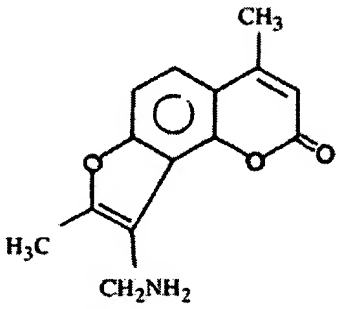
Figure 6: Contamination control procedure based upon formation of pyrimidine dimers (TT or CC) following exposure to ultraviolet light. Excision of thymine dimers is not usually performed by Taq polymerase.

Several other techniques based upon the concept of UV crosslinking have been developed. These techniques utilize UV illumination on a larger scale to expose samples and work space to eliminate contamination. One approach uses small bench top chambers (e.g., rectangular or spherical) that contain small (e.g., two 20 watt) UV lamps that shine inside a self-contained work area to provide a contamination-free work space. Another alternative is to install 254 nm UV lights into the ceiling of the laboratory where the IVAR tubes are assembled so that the entire room would be decontaminated. The latter procedure would expose the entire room (work area) overnight with 254 nm UV to provide a contamination-free

environment for use the following morning. Use of this technique would require additional safety precautions such as door interlocks on the UV circuits to protect persons working in the laboratory against UV exposure.



Psoralen



4'-Aminomethyl-4,5'-dimethyl-isopsoralen
(4'AMDMIP)

Figure 7: Structure of psoralen and related isopsoralen, 4 -AMDMIP (4 aminomethyl-4, 5 -dimethyl-isopsoralen). Both are classified as furocoumarins, psoralen as a linear form and isopsoralen as an angular isomer.

2. Pyrimidine. Psoralen Photoadducts by UV: The second photoreaction is based upon the observation that a class of organic heterocyclic species ("psoralens") can undergo photometric crosslinking reactions with DNA when exposed to ultraviolet light. [17] Psoralen and isopsoralen (Figure 7) interact with dsDNA by intercalating between stacked base pairs. When illuminated with UV light, psoralen can form either monoadducts or can bifunctionally attack both strands to form interstrand derivatives. By contrast, isopsoral (IP) can only form a monoadduct with a single adjacent pyrimidine (C or T). Although psoralen photochemistry has been exploited to chemically sterilize solutions containing DNA viruses, a method using an isopsoralen to control IVAR carryover was just recently described. [18] Several structural analogues of isopsoralen were examined for their ability to crosslink with pyrimidine bases (C and T) in DNA strands. One of the isopsoralens (4'- AMDMIP) was eventually selected and the present contamination control scheme utilizes this particular isopsoralen (Figure 8).

   The isopsoralen-based contamination procedure works this way: The IVAR reagents are assembled and a mastermix is prepared. A small amount of IP is added along with the sample and the PCR is performed as described (Note: The isopsoralen is NOT incorporated into the amplicon during the main amplification reaction.) IMMEDIATELY following the IVAR (PCR), the still-sealed PCR tubes are placed inside a light-tight chamber and irradiated with UV light (300-400 nm). The isopsoralen then forms intrastrand adducts with random pyrimidine residues located along each strand. Because of its structure, isopsoralen does NOT form interstrand adducts, therefore each of the two individual derivatized DNA strands can

independently undergo hybridization reactions. [18] It should be emphasized that since the isopsoralen method is also a photoreaction, only a statistical fraction of the total number of available pyrimidines (C and T) will be modified during the illumination by UV light.

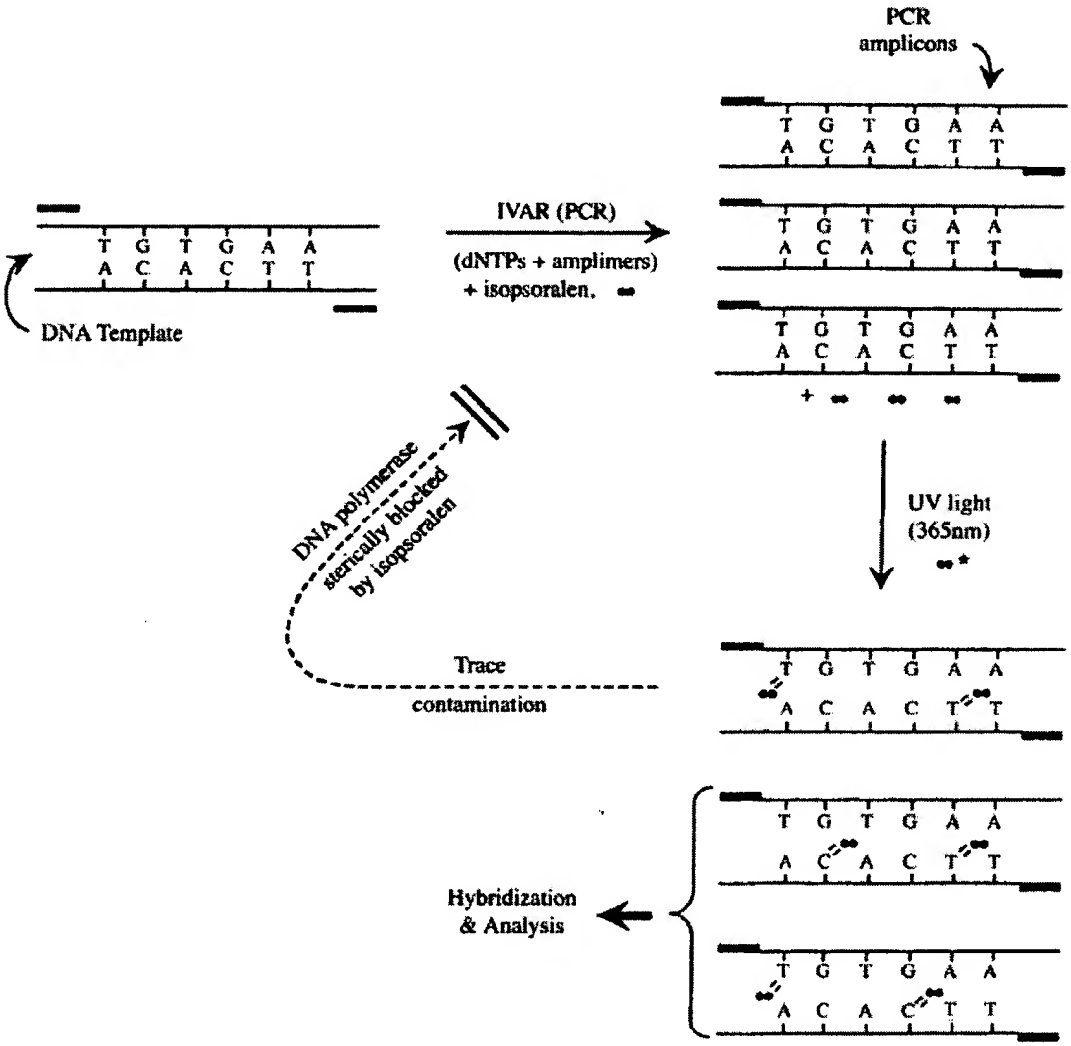


Figure 8: Contamination control technique that uses photoreaction of isopsoralen with pyrimidines (C and T). The isopsoralen species does not participate in the actual PCR reaction itself, but reacts only after it is activated by exposure to UV light. The adduct reaction is a statistical process and not all pyrimidines are modified in each separate amplicon.

A number of factors influence the extent of photoadduct formation, including intensity and wavelength of UV light, concentration of isopsoralen, amount of DNA present, and duration of UV exposure.[8] Although these variables would likely need to be optimized for each amplicon to be controlled, a consistent set of parameters could probably be chosen for most amplicons. The hybridization capability of the derivatized strands has recently been demonstrated using templates prepared from HIV. [19] The primary cost of performing this technique is the purchase of the UV Chamber ($1495.00) and the isopsoralen reagents which are $1.50 per PCR tube. Although this procedure appears to be useful as a contamination control technique for most of the IVAR including the PCR, LCR, RCR, and 3SR, its performance has yet to be thoroughly examined.

GENERAL CONTAMINATION CONTROL PROCEDURES

Besides using the techniques which have been described, there are some basic precautions one should observe to control contamination by IVAR amplicons. [5-7] Detailed explanations of several are listed below:

A. **Laboratory Construction:** Once the decision has been made that an IVAR will be utilized in the laboratory, at least two separate rooms should be designated for IVAR use alone. [5-7] One room (PRE-IVAR) should be designated for preparation of IVAR reagents and samples. UNDER NO CIRCUMSTANCES should POST-IVAR tubes ever be opened or manipulated in this room. A second room for POST-IVAR analysis and manipulation should be established that is physically removed from the PRE-IVAR room.

B. **Environmental Control:** It is preferable that these two rooms (PRE and POST) have independent environment control and not use common ductwork for air conditioning. If desired, additional modification to the laboratories can be undertaken to further control amplicon contamination. For instance, both the PRE-IVAR room and the POST-IVAR can be equipped with air-lock doors. Alternatively, PCR reagents, PCR master mixes and samples ready for cycling can be prepared in laminar flow hoods located away from other laboratory areas (i.e., "clean rooms").

C. **Laboratory Equipment and Personnel:** Each laboratory (PRE and POST) has equipment that is unique to that laboratory (i.e., equipment that is not shared or moved between the two laboratories.) This includes small table-top centrifuges, pipettors, water baths, tube racks, etc., as well as disposable items such as pipet tips, disposable gloves, disposable pipets, etc. [5,6] Movement of persons between laboratories is also

restricted so that one individual ideally works in only one laboratory during a normal workday and does not walk periodically between both locations. Persons who do walk between these two laboratories should not wear the same labcoat or gloves in both locations. Color coded lab jackets may be useful for this purpose. It may also be necessary for individuals to wear a disposable mop cap and face mask when assembling PCR reagents. A recent report traced contamination to a laboratory person who was not protected in this manner. [4] Another report suggests that DNA amplicon contamination may also be minimized by regular use of 10% bleach on exposed surfaces such as bench counters, hoods, centrifuges, etc. [20]

D. **Flow of Samples:** Reagents to be used for IVAR reactions and samples that may contain IVAR templates should be prepared in the PRE-IVAR laboratory ONLY. Long-term storage of these items should be restricted to this laboratory. [7] It is preferable to have the device used to facilitate the IVAR reaction ("temperature cycler") located in the POST-IVAR laboratory in case the tubes are accidentally opened following completion of the IVAR. POST-IVAR tubes should be opened ONLY in the POST-IVAR laboratory and nowhere else. All reagents or materials used for analyzing IVAR amplicon(s) should be disposed of in waste containers that are clearly labeled as originating from the POST-IVAR laboratory. These items should include agarose or acrylamide gels, blotting paper, hybridization solutions, wrapping materials, IVAR tubes, disposable pipets and wash solutions.

SUMMARY

Control of in vitro amplification reaction products is becoming an increasing concern for molecular genetics laboratories in general and for diagnostic molecular pathology laboratories in particular. What may happen within the next several years is that laboratory accreditation organizations such as the CAP (College of American Pathologists) will issue guidelines for clinical laboratories to adhere to in order to maintain certification. How robust these contamination control procedures will be and which will be more compatible with a particular IVAR is not yet known. The objective of maintaining a molecular pathology laboratory in a contamination-free state is clearly necessary. Which methods to select and how to best implement them is undoubtedly the challenge for diagnostic laboratories working in this new field.

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Biography of Theodore E. Mifflin

Theodore E. Mifflin, Ph.D., DABCC, is an Associate Professor of Pathology at the University of Pittsburgh Medical Center, Pittsburgh, PA. He received a B.S. in Chemistry from Weber State University in Ogden, Utah and later a Ph.D. in physical biochemistry from Utah State University in Logan. He was a postdoctoral fellow at the University of Virginia Medical Center from 1983 to 1986 and a Research Assistant Professor at the University of Virginia from 1986 to 1990. In 1991 he accepted his current position at the University of Pittsburgh. Dr. Mifflin directs a molecular genetics laboratory that is part of the Molecular Diagnostics Division within the Department of Pathology. He is also a consultant to Molecular BioProducts, Inc.

About Molecular BioProducts, Inc.

Founded in 1978, Molecular BioProducts, Inc., has been a manufacturer of innovative products designed to eliminate contamination concerns for researchers performing PCR. Now, with a line of patented and proprietary products that are recognized and respected throughout the industry, MBP has become the leader in contamination control products for molecular biology. To further assist you and your lab, MBP is pleased to provide this overview to contamination control in the PCR laboratory. It was the comments and questions of researchers around the world that prompted us to contact Dr. Theodore Mifflin and develop this manual. In it, Dr. Mifflin shares his many years of experience and expertise in a concise and informative way and for this, we owe many thanks.

Our commitment to helping researchers prevent contamination is represented in our line of quality products and in the publication of this booklet, each of which can be used to increase the accuracy and reliability of your research. The valuable information in this report can help solve many of the common problems associated with cross-contamination, but we invite you to contact us with any additional questions or problems that you are facing in the laboratory. We thank you for your continued support and look forward to serving you in the future.

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MEDICAL WRITINGS

"Sensitivity" and "Specificity" Reconsidered: The Meaning of These Terms in Analytical and Diagnostic Settings

Alfred J. Saah, MD, MPH, and Donald R. Hoover, PhD, MPH

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Imprecise usage of the terms "sensitivity" and "specificity" produces confusion in the diagnostic use of sophisticated laboratory test results."Analytical sensitivity" represents the smallest amount of substance in a sample that can accurately be measured by an assay. "Analytical specificity" refers to the ability of an assay to measure one particular organism or substance, rather than others, in a sample. An assay's analytical sensitivity and analytical specificity are distinct from that assay's clinical diagnostic sensitivity and diagnostic specificity. "Diagnostic sensitivity" is the percentage of persons who have a given disorder who are identified by the assay as positive for the disorder. High analytical sensitivity does not guarantee acceptable diagnostic sensitivity. "Diagnostic specificity" is the percentage of persons who do not have a given condition who are identified by the assay as negative for the condition. False-positive reactions occur because of sample contamination and diminish the diagnostic specificity of the assay. The terms "sensitivity" and "specificity" should be used with the requisite adjectives because the "diagnostic" and the "analytical" meanings of these terms are very different.

The terms "sensitive" and "specific" are used in many different contexts. In fact, in a discussion of molecular assays [1], the term "ultrasensitive" was introduced to describe an assay that offered "more sensitivity" than its predecessor. Most health care providers have at least an intuitive notion of these concepts, but the vernacular of test sensitivity and specificity has become more complex and seemingly more contradictory as the use of molecular assays has become more widespread [2]. Thus, difficulties arise when the clinical performance of an assay does not seem to meet expectations. For instance, as many as 2% to 15% of persons who are documented as being seropositive for human immunodeficiency virus type 1 (HIV-1) infection have negative results on the exquisitely sensitive polymerase chain reaction (PCR) assay [3-5]. Understandably, confusion arises when a "highly sensitive" [6] assay often misses the diagnosis or when a "highly specific" test gives a false-positive result [7, 8].

The problem is that any given laboratory test has not one but two kinds of sensitivity and specificity: analytical and diagnostic. Despite important differences between analytical sensitivity and diagnostic sensitivity, these terms are used interchangeably in the clinical setting without being distinguished by their respective adjectives. The same is true for analytical specificity and diagnostic specificity. Understanding the different meanings of these terms is key to properly requesting and interpreting diagnostic test results.

Analytical and Diagnostic Sensitivity

Any assay that purports to measure a given substance has inherent characteristics that are described as "analytical" to distinguish them from the assay's "diagnostic" characteristics [9]. The analytical sensitivity of an assay is that assay's ability to detect a low concentration of a given substance in a biological sample, whether that substance is blood glucose or HIV-1 proviral DNA. This type of sensitivity is expressed as a concentration (for example, in mg/dL or in gene copies/50 million cells) [10]. The lower the detectable concentration, the greater the analytical sensitivity. Synonyms for analytical sensitivity include "limit of detection" and "minimal detectable concentration." Analytical sensitivity may also be expressed in terms of an assay's ability to detect a change in concentration. The smaller the detectable change, the greater the analytical sensitivity.

Analytical sensitivity is determined in one of two ways: empirically, by testing serial dilutions of specimens with a known concentration of the target substance; or statistically, by testing multiple negative specimens and using 2 or 3 standard deviations above the mean as the lower limit of detection. With either method, the goal is to determine the detection limit of the assay [11]. For molecular assays, it is logical to use the empirical method to determine the detection limit.

When an assay is applied to a population to detect a condition or disease, diagnostic sensitivity becomes relevant. The diagnostic sensitivity of a test is the test's ability to detect persons with the condition of interest in a population or group and is expressed as a proportion or percentage: the number of persons who have both the condition and a positive test result divided by the number of persons who have the condition. Diagnostic sensitivity often has more to do with the ability to obtain the target substance in a processed sample from a person who has the condition than with the ability to detect very low concentrations of a substance. If the target substance is not in the processed sample because of vagaries of sampling or processing, an assay with perfect analytical sensitivity still fails to give a positive result.

Polymerase chain reaction is a technologically advanced assay in which a small amount of DNA (very low numbers of copies of a gene or gene fragment) is amplified to make its detection feasible. Since it was invented 11 years ago, PCR has gained wide applicability because the amplification process has been automated [12-14]. It has been used to identify *Borrelia burgdorferi* [15] sequences in synovial fluid and *Treponema pallidum* [16] in brain tissue. Additionally, by altering methods in assay performance, quantitation of an infectious agent (such as HIV-1 DNA or viral RNA) has become routine. In some cases, PCR has greater potential usefulness than do the previous serologic, antigen-detection, or culture-based assays designed to detect various agents or conditions.

However, sizable proportions of persons with documented diseases or infections have negative results on PCR when this assay is used for diagnosis [4, 6, 17-20]. This occurs because the target DNA may be missing from a sample obtained from an infected person. Even with infinite analytical sensitivity, PCR cannot detect the target DNA if that DNA is absent. A similar problem arises when blood culture is used to diagnose bacterial endocarditis. Only one pathogenic organism needs to be in the culture bottle that is obtained and processed from the patient in order for a diagnosis to be yielded. However, capturing and successfully processing that organism may be challenging because its presence in any given sample is unlikely.

The frequent subordination of diagnostic sensitivity to analytical sensitivity is exemplified by the dissonant message conveyed in the following sentence [21], which was written to explain why false-negative results are seen on PCR assays for HIV: "Absence of virus rather than false negativity may explain some of these results." If the patient is truly infected with HIV, as manifested by serologic reactivity, then a negative result on PCR is falsely negative if it is used to diagnose HIV infection. From a diagnostic point of view, it does not matter that the absence of virus in the reaction vessel explains the negative results: The results remain falsely negative and frustrate the clinician's attempt at diagnosis.

False-negative results on PCR or other amplification assays also occur when primer sequences are not properly complementary to the target molecules or when primers fail to bind and amplification fails despite the presence of HIV DNA [3, 22]. This phenomenon also occurs in certain viral load assays for HIV RNA and is due to genetic

intraspecies variation in HIV-1 [23]. Amplification failure may also result from the inhibition of Taq polymerase [24].

Analytical and Diagnostic Specificity

Analytical specificity is the ability of an assay to exclusively identify a target substance or organism rather than similar but different substances (insulin rather than proinsulin; HIV-1 rather than HIV-2) in a sample or specimen. In PCR, the primers for the HIV-1 proviral genome are highly "specific"; that is, they do not measure the proviral DNA of retroviruses other than HIV-1. When an assay is analytically nonspecific, it often produces a positive result when the specimen is truly negative for the exact agent being sought. This problem also diminishes diagnostic specificity, which is the ability of an assay to correctly identify a person who does not have the disease in question.

However, because an assay such as PCR is extraordinarily sensitive analytically, the slightest exogenous contamination with previously amplified HIV-1 DNA (carryover) causes a false-positive test result in an assay that is "very highly specific" (analytically). The result, therefore, may be that analytical and diagnostic specificity diverge because the assay maintains its very high analytical specificity but becomes diagnostically misleading because of external contamination.

Given the potential for contamination due to carryover, a clinical dilemma may arise when the only indication of HIV-1 infection is a positive result on PCR. There is no standard by which to measure the true state of infection, except for the passage of time and the emergence of HIV-specific antibodies. The problem of carryover is one issue that may limit the general applicability of PCR results for HIV-1 in the diagnostic and screening setting ([25]; U.S. Food and Drug Administration Conference on the Feasibility of Genetic Technology to Close the HIV Window in Donor Screening, Silver Spring, MD, 1994).

Yet another issue affects the diagnostic specificity of PCR for detecting true as opposed to "perceived" infection. The PCR assay may detect DNA fragments that do not represent intact organisms capable of reproducing or causing disease [15, 26]. Here again, the test has not lost analytical specificity but gives diagnostically incorrect results.

Diagnostic Predictive Value and Clinical Relevance

Sensitivity and specificity define the operating characteristics of an assay, but it is the predictive value (positive or negative) of the assay that is generally of diagnostic importance to clinician and patient. Positive predictive value is the probability that a person whose test result is positive truly has the disease or condition of interest (that is, of every 100 patients who have positive test results, the number of patients who have the disease). Negative predictive value is the probability that a person whose test result is negative does not have the disease of interest (that is, of every 100 patients who have negative test results, the number of patients who do not have the disease). Strong diagnostic sensitivity improves negative predictive values, and strong diagnostic specificity improves positive predictive values (regardless of analytical sensitivity and analytical specificity). For example, if a test has perfect diagnostic sensitivity and perfect diagnostic specificity, then all persons who have positive test results have the disease and all persons who have negative test results do not. Accordingly, assays that have very high analytical sensitivity and specificity but have low diagnostic sensitivity and specificity have a poor diagnostic predictive value. For example, if an "ultrasensitive" assay (one with high analytical sensitivity) often gives positive results because of contaminated samples, then a positive test result may not strongly suggest disease. On the other hand, if an "ultrasensitive" assay fails to give a positive result because the patient samples do not contain the target molecules or if heterologous molecules are improperly measured because of very high analytical specificity, then a negative test result does not guarantee the absence of disease.

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Imprecise usage of the terms "sensitivity" and "specificity" produces confusion in the diagnostic use of laboratory tests, particularly certain molecular assays, such as PCR. Important distinctions exist between the analytical and diagnostic use of these terms. The analytical sensitivity of an assay represents the smallest amount of a substance that can be accurately measured in a biological sample; analytical specificity is the assay's ability to measure a particular organism or substance, rather than another, in a sample. These characteristics are distinct from diagnostic sensitivity and specificity. In the clinical setting, diagnostic sensitivity is defined by the percentage of persons who have the disorder of interest who have positive results on the assay. Although one might expect that an analytically sensitive assay should more readily identify those persons, the ability to measure a very small quantity of a substance does not always translate into high diagnostic sensitivity. This apparent contradiction results from the shortcomings of sampling a very small volume, variations in the clinical spectrum of disease, and possible difficulties with specimen preparation and technical performance of the assay. Diagnostic specificity is defined by the percentage of persons who do not have the condition of interest who have negative results on the assay. False-positive reactions diminish the diagnostic specificity; these reactions may be particularly likely to occur in molecular assays as a result of contamination with amplified material from other reactions (carryover).

Assays with extraordinarily high analytical sensitivity and specificity will almost certainly not perform at these very high levels diagnostically. Thus, great care is required when test results are being interpreted for patient management. Clinicians must be cautious when the terms "sensitivity" and "specificity" are used without the requisite adjectives by laboratory-based colleagues or in advertisements for laboratory services or test kits, because the "diagnostic" and "analytical" meanings of these terms are not the same.

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1: Nature. 1989 May 18;339(6221):237-8.

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- Nature 1989 Jun 8;339(6224):490.

Avoiding false positives with PCR.

Kwok S, Higuchi R.

Department of Infectious Diseases, Cetus Corporation, Emeryville, California 94608.

The exquisite sensitivity of the polymerase chain reaction means DNA contamination can ruin an entire experiment. Tidiness and adherence to a strict set of protocols can avoid disaster.

PMID: 2716852 [PubMed - indexed for MEDLINE]

1: Ugeskr Laeger. 1993 May 31;155(22):1708-12. Related Articles, Links

[Polymerase chain reaction: use in microbiological diagnosis]

[Article in Danish]

Lisby G.

Klinisk mikrobiologisk afdeling, Kobenhavns Amts Sygehus i Herlev.

The discovery of the polymerase chain reaction (PCR) enables the detection of just a few target gene copies present in almost every kind of tissue. The PCR technique uses two separated specific DNA-sequences for identification of a desired genetic sequence. This identification is followed by an almost unlimited production of the specific target gene sequence by a heat stable DNA-polymerase. The PCR technique will revolutionize several diagnostic areas, and especially the identification of virus, fungi and slowly growing bacteria will benefit from this new genetic technology. The impressive sensitivity is, however, the greatest pitfall of the technique, as just a few contaminating DNA fragments can initiate a false positive result. Great care is therefore needed when designing a PCR laboratory, as well as high demands upon the motivation and technical skills of the personnel involved. PCR is so far limited to research, but is expected to be released for routine diagnostic purposes in 1993.

Publication Types:
- Review

PMID: 8317014 [PubMed - indexed for MEDLINE]

1: J Acquir Immune Defic Syndr. 1990;3(5):517-24. Related Articles, Links

Detection and characterization of HIV-1 by polymerase chain reaction.

Krone WJ, Sninsky JJ, Goudsmit J.

Department of Virology, University of Amsterdam, Academic Medical Center, The Netherlands.

Recently a new technique, the polymerase chain reaction (PCR), has been used for the detection and characterization of HIV-1 proviral DNA and viral RNA. These reports support the notion that the PCR is more sensitive and specific than other established HIV-1 detection techniques. However, due to its extreme sensitivity, the PCR is highly susceptible to contamination, resulting in false positive results. To avoid contamination, strict rules on sample preparation and pre- and post-PCR handling are required. Confirmation of both positive and negative PCR results by independent techniques is not always feasible, and, therefore, optimal PCR conditions, inclusion of control samples, repetition of results, and confirmation of specificity by hybridization are required. The choice of the material from which HIV-1 is amplified, the primers used for amplification as well as the PCR conditions will determine what is actually amplified.

PMID: 2324945 [PubMed - indexed for MEDLINE]

1: J Virol Methods. 1991 Aug;33(3):375-82. Related Articles, Links

Eliminating PCR contamination: is UV irradiation the answer?

Fox JC, Ait-Khaled M, Webster A, Emery VC.

Division of Communicable Diseases, Royal Free Hospital School of Medicine, London, U.K.

The sensitivity of the polymerase chain reaction (PCR) can mean that even very low levels of contamination with the target DNA will result in a positive signal. At present this aspect is a major limitation in the use of PCR as a routine diagnostic method. By exposing PCR reagents to UV light, contaminating DNA can be inactivated, thus providing an opportunity to eradicate false positive reactions. UV irradiation was applied to PCR systems used for the detection of human cytomegalovirus (CMV) and human immunodeficiency virus (HIV) and shown to be effective in eradicating both laboratory encountered contamination and plasmid DNA (below 100 pg) added to PCR systems prior to UV exposure. The sensitivity of a PCR system to amplify the long terminal repeat (LTR) sequence of HIV-1 was not affected by the irradiation procedure; however, the ultimate sensitivity of a PCR system for the amplification of an early gene promotor sequence of the CMV genome was reduced 1000-fold. UV irradiation did not affect the size of the PCR product as determined by strand separating polyacrylamide gel electrophoresis of a 32P-labelled amplimer. Thus, a simple pre-exposure to UV light would seem a worthwhile step to incorporate into PCR protocols provided that the effects on sensitivity have been determined empirically for each PCR system.

PMID: 1664435 [PubMed - indexed for MEDLINE]

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1: Clin Chem Lab Med. 2000 Feb;38(2):87-91. Related Articles, Links

Standardization and quality control of PCR analyses.

Burkardt HJ.

Roche Diagnostics Switzerland, Molecular Systems, Rotkreuz. Hans-Joachim.Burkardt@roche.com

In the very beginning of polymerase chain reaction (PCR) tests entering the field of diagnosis of infectious agents, the introduction of this technology into routine diagnosis was hampered by its frequent tendency to create false-positive results because of contamination. This problem is now widely solved by the introduction of the uracil-N-glycosylase (UNG) anticontamination technology. However, care must still be taken to avoid other sources of producing false positive results. They might additionally derive from human error and/or insufficient PCR amplification and detection protocols. A special case lies in the fact that PCR also amplifies DNA from dead organisms rendering a result diagnostically correct as positive, but clinically as false-positive. In PCR, as in any other diagnostic test, the risk of creating a false-negative result also exists. In such a case, the most probable source besides human error, low target or poor amplification and detection protocols is an inhibition caused by interfering substances in a patient's sample. Strategies to recognize and overcome this issue are discussed in this article. Finally a few results from quality control studies on amplification technologies in the diagnosis of infectious agents are reviewed.

Publication Types:
- Review

PMID: 10834394 [PubMed - indexed for MEDLINE]

1: Mol Cell Probes. 1991 Aug;5(4):241-59.

The polymerase chain reaction: a new tool for the understanding and diagnosis of HIV-1 infection at the molecular level.

Coutlee F, Viscidi RP, Saint-Antoine P, Kessous A, Yolken RH.

Departement de Microbiologie et Maladies Infectieuses, Hopital Notre-Dame, Montreal, PQ, Canada.

The polymerase chain reaction (PCR) is at present the most powerful analytical tool for detection of specific nucleic acid sequences. The method is based on the in vitro amplification of DNA segments before detection with conventional hybridization techniques or visualization following electrophoresis and staining. The current diagnostic methods for HIV-1 do not allow easy identification of subgroups of infected patients including infants born to seropositive mothers, individuals with delayed serological responses to the virus, infected patients with indeterminate serology results, and patients with dual retroviral infections. Furthermore, response to antiviral therapy cannot be evaluated with serological assays. The rationale for applying PCR in those situations is elaborated here. The applications of this technique for HIV-1 as a diagnostic test and for the understanding of the pathogenesis of this retrovirus are described. Potential limitations of this technique for diagnostic purposes include mainly the possibility of false-positive results due to contamination and false-negative reactions caused by Taq polymerase inhibition. Non-isotopic means for detection of amplified products have been described and should allow for a wider application of this technology. Modifications of PCR which make use of internal standards seem promising for quantitative analysis of nucleic acids. PCR has great potential for viral diagnosis but still requires further studies and better characterization.

Publication Types:
- Review

PMID: 1791846 [PubMed - indexed for MEDLINE]